DEFINING MECHANISMS THAT DETERMINE THE LEVELS OF DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

Margaretha Bester

Disssertation presented for the degree of Master of Science at Stellenbosch University

Promoter: Prof. TC Victor

Co-Promoter: Dr. R Johnson

December 2009

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, orginal work, that I am the owner of the copyright thereof (unless to the extent explicity otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 3 November 2009

SUMMARY

Varying levels of Rifampicin (RIF) resistance in closely related clinical *Mycobacterium tuberculosis* isolates and *in vitro* generated mutants question the dogma that non-synonymous single nucleotide polymorphisms in the *rpoB* gene are the only mechanism explaining RIF resistance. This study aimed to identify biological mechanisms that define the level of RIF resistance in two closely related clinical *M. tuberculosis* isolates using proteomic, transcriptomic and genomic approaches. Two dimensional electrophoresis revealed an increase in the abundance of numerous membrane proteins in response to RIF at the critical concentration of 2μg/ml. Fourty-one of these proteins were identified by mass spectrometry and could be grouped according to their cellular function (Energy metabolism, degradation, biosynthesis of cofactors, metabolic groups and carriers, lipid biosynthesis, central intermediate metabolism, synthesis and modification of macromolecules, chaperone/heat shock proteins). The identification of proteins responsible for ATP synthesis (atpA and atpH) suggests an ATP requirement to combat the toxic effect of RIF. These proteins are components of the FoF1 ATP synthase an enzyme which is involved in the oxidative phosphorylation pathway that generates ATP in the cell. QRT-PCR confirmed the up regulation of the transcription of the *atpA* and *atpH* genes in response to RIF, while DNA sequencing failed to identify mutations that could define the rate of transcription.

To explain our findings we proposed that RIF induces a toxic response leading to the up regulation of a number of genes. The induction of metabolic enzymes, such as the FoF1 ATP synthase provides energy to activate ATP dependant mechanisms, including membrane ABC transporters. These ABC transporters actively pump RIF out of the cell thereby lowering the intracellular concentration of RIF to below its binding concentration with the rpoB protein leading to RIF resistance. Inhibition of efflux by the efflux pump inhibitors reserpine and verapamil leads to an accumulation of RIF within the cell and concurrent binding of RIF to rpoB, leading to inhibition of transcription and cell death (ongoing research in our laboratory). Similarly, we propose that the recently identified diarylquinoline compound (TMC207) inhibit ATP synthesis, thereby depleting the energy source necessary for active efflux. This will lead to an accumulation of anti-TB drug within the cell and subsequent cell death. In summary, this study provides the first evidence to suggest that the evolution of RIF resistance is a dynamic process involving a cascade of adaptive events which leads to a bacterial growth state where hydrophobic compounds are actively extruded from the cell. This has important ramifications for the treatment of RIF resistant TB and

supports the need for the development of anti-TB drugs that target both efflux and ATP synthesis to improve the treatment outcome of MDR-TB and XDR-TB.

OPSOMMING

Verskillende vlakke van Rifampisien (RIF) weerstandigheid, in naby verwante *Mycobacterium tuberculosis* kliniese isolate en *in vitro* mutante, bevraagteken die dogma dat nie-sinonieme enkel nukleotied polimorfismes in die *rpoB* geen die enigste verklaarbare meganisme vir RIF weerstandigheid is. Die doel van hierdie studie was om deur ‘n proteomiese, transkriptomiese en genomiese benadering, biologiese meganismes te identifiseer wat die vlakke van RIF weerstandigheid in twee naby verwante kliniese *M. tuberculosis* isolate bepaal. Twee dimensionele elektroferese het gevind dat daar ‘n verhoging in die hoeveelheid van verskeie proteïne is wanneer die isolate aan RIF by die ‘n kritiese konsentrasie van 2µg/ml blootgestel is. Massa spektrometrie het 41 van hierdie proteine geïdentifiseer en die proteïne kan gegroepeer word in verskeie sellulêre funksies (Energie metabolism, degradering, biosintese van kofaktore, metaboliese groepe en draers, lipied biosintese, sentrale intemediêre metabolisme, sintese en modifisering van makromolekules, en “chaperone/heat shock” proteine). Die identifisering van proteïne verantwoordlik vir ATP sintese (atpA en atpH) stel voor dat ATP belangrik is om die toksiese effek van RIF te ontwyk. Hierdie proteïne is komponente van die FoF1 ATP sintase ensiem wat betrokke is in die oksidatiewe fosforilerings pad en wat lei tot die generering van ATP in die sel. Kwantitatiewe QRT-PCR het bevestig dat hierdie gene, *atpA* en *atpH*, opgereguleer word nadat die bakterium aan RIF blootgestel is. In teen deel kon DNA volgorde bepaling nie mutasies identifiseer wat die verandering in geen transkripsie kon verklaar nie.

Om ons bevindings te verduidelik, stel ons voor dat RIF ‘n toksiese effek in die sel induseer wat lei tot die opregulering van verskeie gene. Die indusering van metaboliese ensieme, soos die FoF1 ATP sintase, voorsien energie om ATP afhanklike meganismes, insluitende membraan ABC transporters, te aktiveer. Hierdie ABC transporters pomp RIF aktief uit die sel, wat daarvolgens die intrasellulêre konsentrasie van RIF verlaag tot ‘n konsentrasie laer as die bindings konsentrasie met die rpoB protein en gevolglik lei tot weerstandigheid. Die onderdrukking van membraan pompe wat RIF uit die sel pomp deur middels soos reserpine en

verapamil sal aanleiding gee lei tot akkumulering van RIF in die sel. Die verhoogde RIF in die sel versoorsaak dat RIF aan die rpoB protein gebind bly sodat dit transkripsie inhibeer, wat dan aanleiding gee tot seldood. (voortgesette navorsing in ons laboratorium). Soortgelyk, stel ons voor dat die onlangs geïdentifiseerde dairylquinoline verbinding (TMC207) ATP sintese inhibeer en daarvolgens die energie bron uitput wat noodsaaklik is vir aktiewe uitpomp van RIF. Dit sal aanleiding gee tot die ophoping van RIF in die sel en gevolglik lei tot seldood.

In opsomming, hierdie studie voorsien die eerste bewys wat voorstel dat die evolusie van RIF weerstandighied ’n dinamiese proses is. Dit sluit ‘n kaskade van aanpasbare gebeurtenisse in wat lei tot ‘n bakteriële groei fase waar hidrofobiese verbindings aktief uit die sel gedryf word. Dit het rampspoedige gevolge vir die behandeling van RIF weerstandige TB en ondersteun die noodsaaklikheid om teen-TB middels te ontwikkel wat beide effluks pompe en ATP sintese teiken om die uikoms van behandeling vir MDR-TB en XDR-TB te verbeter.

ACKNOWLEDGEMENTS

This work would not have been possible without the support and encouragement of the following people:

- Prof. Tommie Victor (promoter), Dr Rabia Johnson (co-promoter), Prof. Rob Warren, Dr Gail Louw and Faghri February for their patience, guidance, advice and excellent discussions and suggestions.
- My parents, sisters (Hannelie and Christelle), Pierrie and all my friends for their love and support.
- All my colleagues and friends at the department.
- The Medical Research Council and the Department of Biomedical Sciences for financial support.

Jesus Christus, my Verlosser, aan U kom al die eer.

Now to Him who is able to do immeasurably more than we ask or imagine, according to His power that is at work within us.

Ephesians 3:20

LIST OF ABBREVIATIONS

°C	:	Degree Celsius
µl	:	microlitres
2-DE	:	2-Dimensional gel electrophoresis
ABC	:	ATP binding cassette
AcOH	:	Acetic acid
ADC	:	Albumin dextrose catalase
AM	:	Amikacin
bp	:	base pairs
BSA	:	Bovine serum albumin
CAP	:	Capreomycin
cDNA	:	Complementary DNA
CIP	:	Ciprofloxacin
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonecleotide triphosphate
EMB	:	Ethambutol
ETH	:	Ethionamide
EtOH	:	Ethanol
FQ	:	Fluoroquinolone
g	:	Grams
IEF	:	Isoelectric focusing
INH	:	Isoniazid

IPG	:	Immobilised pH gradient
KAN	:	Kanamycin
KCI	:	Potassium cloride
LAM	:	Latin-American and Mediterranean
LCC	:	Low Copy Clade
LJ	:	Loewenstein Jensen
M. tuberculosis	:	*Mycobacterium tuberculosis*
MALDI-TOF	:	Matrix Assisted Lazer Desorption/Ionization Time of Flight
MATE	:	Multidrug And Toxic compounds Extrusion
MDR	:	Multi Drug Resistant
MeOH	:	Methanol
MFS	:	The Major Facilitator Super family
MIC	:	Minimum Inhibitory Concentration
MIG	:	Master Image Gel
ml	:	millilitres
mM	:	mM
mRNA	:	Messenger RNA
MS	:	Mass spectrometry
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
ng	:	nanograms
nsSNP	:	Non Synonymous SNP
OD	:	Optical density
OFX	:	Ofloxacin

PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PMSF	:	Phenylmethylsulfonyl fluoride
QRT-PCR	:	Quantitative REAL-TIME PCR
RFLP	:	Restriction Fragment Length Polymorphism
RIF	:	Rifampicin
rpm	:	Revolutions per minute
RNA	:	Ribonucleic acid
RND	:	Resistance-Nodulation-cell Division
RRDR	:	RIF Resistance Determining Region
rRNA	:	Ribosomal RNA
SA	:	South Africa
SDS	:	Sodium dodecyle sulphate
SMR	:	Small Multidrug Resistance
SNP	:	Single nucleotide polymorphism
STR	:	Streptomycin
TB	:	Tuberculosis
TBE	:	Tris/Borate/EDTA
TE	:	Tris/EDTA
Tm	:	Melting temperature
Tris	:	Trishydroxymethylaminomethane
U	:	Units
V	:	Volt
WCL	:	Whole cell lysate

XDR	:	Extreme Drug Resistant
ZN	:	Ziehl-Neelsen

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

South Africa, with a Tuberculosis (TB) incidence of 948/100 000 per annum and 15 914 Multi Drug Resistant (MDR) cases reported in 2007, was declared by the WHO as one of the countries with the highest burden of TB (16). MDR-TB is defined as *Mycobacterium tuberculosis (M. tuberculosis)* strains resistant to the two most important first-line anti-TB antibiotics, Isoniazid (INH) and Rifampicin (RIF) (6). Recently Extreme Drug Resistant (XDR) strains were identified and these are currently widespread found in all provinces of South Africa. XDR-TB strains are MDR and in addition are also resistant to any Fluoroquinolone and one of the injectable antibiotics, i.e. Kanamycin, Amikacin and Capreomycin (6). These two forms of highly drug resistant strains is a major public health concern globally. For the past 4 decades no new anti-TB drugs have been developed (9). This raises the concern of a future epidemic of virtually untreatable TB and the need for the development of new drugs to effectively treat all forms of TB (8).

The TB incidence in the Western Cape is an alarming 1005.7/100 000 per annum (3). Approximately 70% of the drug resistant epidemic in the Western Cape are driven by 4 strain families: Beijing/W-like (28%), Low Copy Clade (LCC) (26%), F11 (12%) and F28 (5%) (11). Recently, outbreaks of MDR clones within the Beijing (R220) and LCC (DRF150) families were reported in local communities. Strains belonging to the Beijing R220 genotype are characterized by an IS*6110* insertion at position 3709536 and a -15 $inhA_{C\text{-}T}$ promoter mutation (conferring INH resistance). The DRF 150 genoptype is characterized by a unique spoligotype and RFLP patterns as well as a dinucleotide mutation at position 315 in the *katG* gene (315*gc* → *ca*) (15). These specific strains still transmits successfully, irrespective of the presence of characteristic mutations. Therefore it is suggested that these strains have unique properties which aids in increased transmissibility and drug tolerance.

RIF is considered to be one of the most important front-line drugs used to treat TB. RIF targets and interacts with the beta subunit of the RNA polymerase, hindering RNA synthesis and therefore killing the organism (12). In contrast, resistance to RIF develops through single nucleotide substitutions in the 81bp core region called the RIF Resistance Determining Region (RRDR) of the RNA polymerase gene (*rpoβ*)

(13). These nucleotide substitutions result in structural conformational changes in RNA polymerase, resulting in defective binding of RIF to RNA polymerase. Resistance to RIF can be considered as a marker for Multi Drug Resistance-TB as mono resistance to RIF is rarely seen and is usually accompanied by INH resistance (2,4,7).

RIF resistant characteristics of *M. tuberculosis* have been extensively studied in the laboratory strain, H37Rv, and *in vitro* selected mutants. It is suggested that a direct relationship exist between different nsSNP's causing drug resistance and the level of drug resistance (14,17). It is possible that each nsSNP in the *rpoB* gene alters the dissociation constant of the mutated *rpoB* ($rpoB_{mut}$) RIF complex (Kd^R) in such an manner that this strongly influences the RIF MIC. However, it has been shown that *in vitro* RIF MIC's are highly variable at specific *rpoB* codons when measured among drug-resistant TB clinical isolates (1,10). Furthermore, varying RIF MIC's among *in vitro* generated isogenic RIF-resistant clones (with identical nsSNP's in the *rpoB* gene) have been reported (5). However, the mechanism by which a strain becomes hyper-resistant to RIF is still not known.

It has been shown that Mycobacteria have adapted to implicate other mechanisms, other than chromosomal alterations in anti-TB target genes, to become resistant to anti-TB drugs. These mechanisms and its regulation thereof will be discussed in detail in Chapter 2 of this thesis.

1.2. PROBLEM STATEMENT

Our group (Phd Thesis, Gail E Louw, 2009) observed that RIF MIC's varied in genotypically closely related clinical isolates which share identical IS*6110* genotypes and identical drug resistance causing gene mutations. In these isolates, the RIF MIC's ranged from 5 to 170 μg/ml in 7H9 liquid media. This challenges the dogma that a single nsSNP in the *rpoB* gene defines the level of RIF resistance. Accordingly we suggest that RIF-resistance develops through a stepwise process beginning with an initial nsSNP within the *rpoB* gene, which is followed by either subsequent mutations in other genes or by drug induced gene regulation which modulates the intra-cellular concentration of RIF.

1.3. HYPOTHESIS

We hypothesise that *M. tuberculosis* might develop RIF resistance through other mechanisms and unique pathways due to selective pressure under prolonged exposure to RIF and patient non-compliance. The combination of nsSNP in the *rpoB* gene and these unique mechanisms defines the level of RIF and enables the organism to become hyper-resistant.

1.4. AIMS

The aim of this project is to identify mechanisms which define the level of RIF resistance in *M. tuberculosis.*

1.5. EXPERIMENTAL APPROACH

Proteomic, transcriptomic and genomic methods were used to identify mechanisms which can explain the varying levels of RIF resistance in two closely related clinical isolates.

1.6. REFERENCES

1. **Cummings, M. P. and M. R. Segal**. 2004. Few amino acid positions in rpoB are associated with most of the rifampin resistance in Mycobacterium tuberculosis. BMC.Bioinformatics. **5**:137.

2. **Gillespie, S. H.** 2002. Evolution of drug resistance in Mycobacterium tuberculosis: clinical and molecular perspective. Antimicrob.Agents Chemother. **46**:267-274.

3. Health Systems Trust. Department of Health (TB section). 1. 2007.
Ref Type: Data File

4. **Hoek, K. G., N. C. Gey Van Pittius, H. Moolman-Smook, K. Carelse-Tofa, A. Jordaan, G. D. van der Spuy, E. Streicher, T. C. Victor, P. D. van Helden, and R. M. Warren**. 2008. Fluorometric assay for testing rifampin susceptibility of Mycobacterium tuberculosis complex. J.Clin.Microbiol. **46**:1369-1373.

5. **Huitric, E., J. Werngren, P. Jureen, and S. Hoffner**. 2006. Resistance levels and rpoB gene mutations among in vitro-selected rifampin-resistant Mycobacterium tuberculosis mutants. Antimicrob.Agents Chemother. **50**:2860-2862.

6. **Jones, K. D., T. Hesketh, and J. Yudkin**. 2008. Extensively drug-resistant tuberculosis in sub-Saharan Africa: an emerging public-health concern. Trans.R.Soc.Trop.Med.Hyg. **102**:219-224.

7. **Mokrousov, I., T. Otten, B. Vyshnevskiy, and O. Narvskaya**. 2003. Allele-specific rpoB PCR assays for detection of rifampin-resistant Mycobacterium tuberculosis in sputum smears. Antimicrob.Agents Chemother. **47**:2231-2235.

8. **Murphy, D. J. and J. R. Brown**. 2008. Novel drug target strategies against Mycobacterium tuberculosis. Curr.Opin.Microbiol. **11**:422-427.

9. **Sacks, L. V. and R. E. Behrman**. 2008. Developing new drugs for the treatment of drug-resistant tuberculosis: a regulatory perspective. Tuberculosis.(Edinb.) **88 Suppl 1**:S93-100.

10. **Srivastava, K., R. Das, P. Jakhmola, P. Gupta, D. S. Chauhan, V. D. Sharma, H. B. Singh, A. S. Sachan, and V. M. Katoch**. 2004. Correlation of mutations detected by INNO-LiPA with levels of rifampicin resistance in Mycobacterium tuberculosis. Indian J.Med.Res. **120**:100-105.

11. **Streicher, E. M., R. M. Warren, C. Kewley, J. Simpson, N. Rastogi, C. Sola, G. D. van der Spuy, P. D. van Helden, and T. C. Victor**. 2004. Genotypic and phenotypic characterization of drug-resistant Mycobacterium tuberculosis isolates from rural districts of the Western Cape Province of South Africa. J.Clin.Microbiol. **42**:891-894.

12. **Taniguchi, H., H. Aramaki, Y. Nikaido, Y. Mizuguchi, M. Nakamura, T. Koga, and S. Yoshida**. 1996. Rifampicin resistance and mutation of the rpoB gene in Mycobacterium tuberculosis. FEMS Microbiol.Lett. **144**:103-108.

13. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer**. 1993. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet **341**:647-650.

14. **van Soolingen, D., P. E. de Haas, H. R. van Doorn, E. Kuijper, H. Rinder, and M. W. Borgdorff**. 2000. Mutations at amino acid position 315 of the katG gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of Mycobacterium tuberculosis in the Netherlands. J.Infect.Dis. **182**:1788-1790.

15. **Victor, T. C., E. M. Streicher, C. Kewley, A. M. Jordaan, G. D. van der Spuy, M. Bosman, H. Louw, M. Murray, D. Young, P. D. van Helden, and R. M. Warren**. 2007. Spread of an emerging Mycobacterium tuberculosis drug-resistant strain in the western Cape of South Africa. Int.J.Tuberc.Lung Dis. **11**:195-201.

16. **World Health Organization**. 2007. WHO REPORT 2007 Global Tuberculosis Control Surveilance, Planning, Financing, p. 3-267.

17. **Zaczek, A., A. Brzostek, E. ugustynowicz-Kopec, Z. Zwolska, and J. Dziadek**. 2009. Genetic evaluation of relationship between mutations in rpoB and resistance of Mycobacterium tuberculosis to rifampin. BMC.Microbiol. **9**:10.

CHAPTER 2

LITERATURE REVIEW

MECHANISMS THAT DEFINE THE LEVEL OF DRUG RESISTANCE IN MYCOBACTERIA

Introduction

Bacteria can develop resistance to antibiotics through spontaneous chromosomal mutations followed by natural selection upon antibiotic exposure. The genetic information generated by these chromosomal alterations will be inherited by their progeny (127). Bacteria also have the ability to transfer this genetic information to other bacteria of the same or distant-related species through horizontal transfer (or plasmid exchange) (13). Horizontal gene transfer can be achieved by transduction, transformation or bacterial conjugation (96).

Mycobacterium tuberculosis (M. tuberculosis), the causative bacteria of Tuberculosis (TB), does not contain plasmids (134). This pathogen develops resistance to numerous antibiotics through the acquisition of spontaneous chromosomal mutations in a variety of genes (Table 2.1). Drug resistant mutants are subsequently selected as a result of ineffective treatment or non-compliance by the patient (59). Other factors promoting the development of drug resistance include the use of low quality drugs, mal absorption and the failure to use standardized short-course chemotherapy (52,102). Amplification of resistance results from the ineffective management of the TB control program, improper diagnosis, and diagnostic delay (131).

Four groups of drug resistance have been described; Mono resistance is defined as resistance to a single anti-TB drug, multiple resistance is defined as resistance to more than one of the first line drugs, but susceptible to Isoniazid (INH) and/or Rifampicin (RIF), multi-drug resistant (MDR) TB is defined as *M. tuberculosis* strains that are resistant to the two most important first-line antibiotics, Isoniazid (INH) and Rifampicin (RIF), whereas extensively drug resistant (XDR)-TB is defined as MDR-TB with addition resistance to any Fluoroquinolone (FQ) and one of the injectable antibiotics i.e. Kanamycin, Amikacin and Capreomycin (60). More recently a further definition has been described, namely totally drug resistant TB (TDR) which is defined as MDR strains resistant to all second-line drugs (125).

Drug resistance in Mycobacteria is either intrinsic (natural) or acquired (chromosomal mutation). Intrinsic resistance is attributed to; i) the permeability of the lipid-rich hydrophobic cell wall which consists of

mycolic acids, arabino and peptidoglycans (26,58), ii) expression of active efflux systems (36,37), iii) the modification and inactivation of the drug, iv) absence of enzymes required to activate the prodrug, v) alteration in the structure of the drug target (reduced affinity of the drug for the target), and vi) altered expression of the drug target (Figure 2.1). Acquired resistance is attributed to chromosomal changes in regulatory domains and target genes leading to; i) the inability activate the prodrug, ii) alteration of the drug target (reduced affinity of the drug for the target), and iii) alteration in expression of drug target (Figure 2.1). Both intrinsic and acquired mechanisms define the minimum inhibition concentration (MIC) of a drug required to kill 99% of the bacterial population.

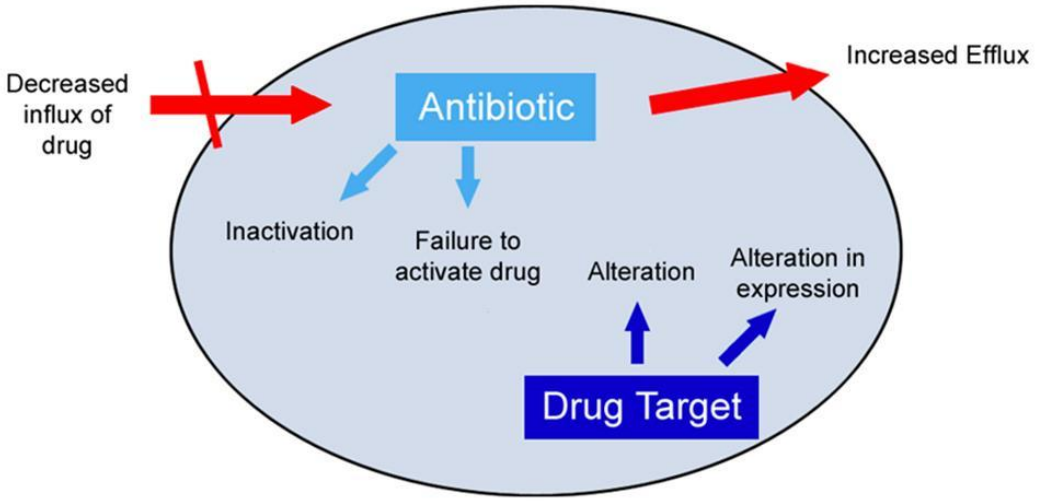

Figure 2.1: Mechanisms of drug resistance in mycobacteria.

The molecular mechanisms of acquired drug resistance in mycobacteria have been studied extensively and several reviews have been published on this topic (59,99,104). Table 2.1 summarizes the genes involved in conferring acquired resistance to both first and second line anti-TB drugs. This review aims to describe mechanisms which affect the level of intrinsic and acquired drug resistance.

GENERAL OVERVIEW OF GENE REGULATION

Gene regulation in bacteria is mediated by either proteins that act as transcriptional regulators (repressors or activators) or modulation of the RNA structure (also called transcription attenuation) (25). Transcriptional attenuation is the process where mRNA is alternatively folded in the leader region upstream of the coding sequence. This results in premature attenuation of transcription. Bacteria also make use of operon systems to coordinately transcribe sets of genes encoding functionally related proteins. These genetic mechanisms can control metabolic events in response to environmental conditions which may include exposure to drugs (63). An operon generally consists of a promoter, operator, two or more structural genes and a repressor gene. The structural genes are regulated by an allosteric repressor molecule transcribed from the repressor gene. This repressor molecule interacts with a regulatory element, the operator, and represses transcription until it interacts with a chemical inducer (Figure 2.2A) (128). This interferes with RNA polymerase which binds to the promoter region and transcription of the structural genes is repressed. However, in the presence of an inducer molecule, it binds to the repressor protein and causes an allosteric conformational change of the repressor protein (Figure 2.2B). This reduces the affinity of the repressor protein for the operator, which relieves the negative regulation, allowing the RNA polymerase to transcribe the structural genes.

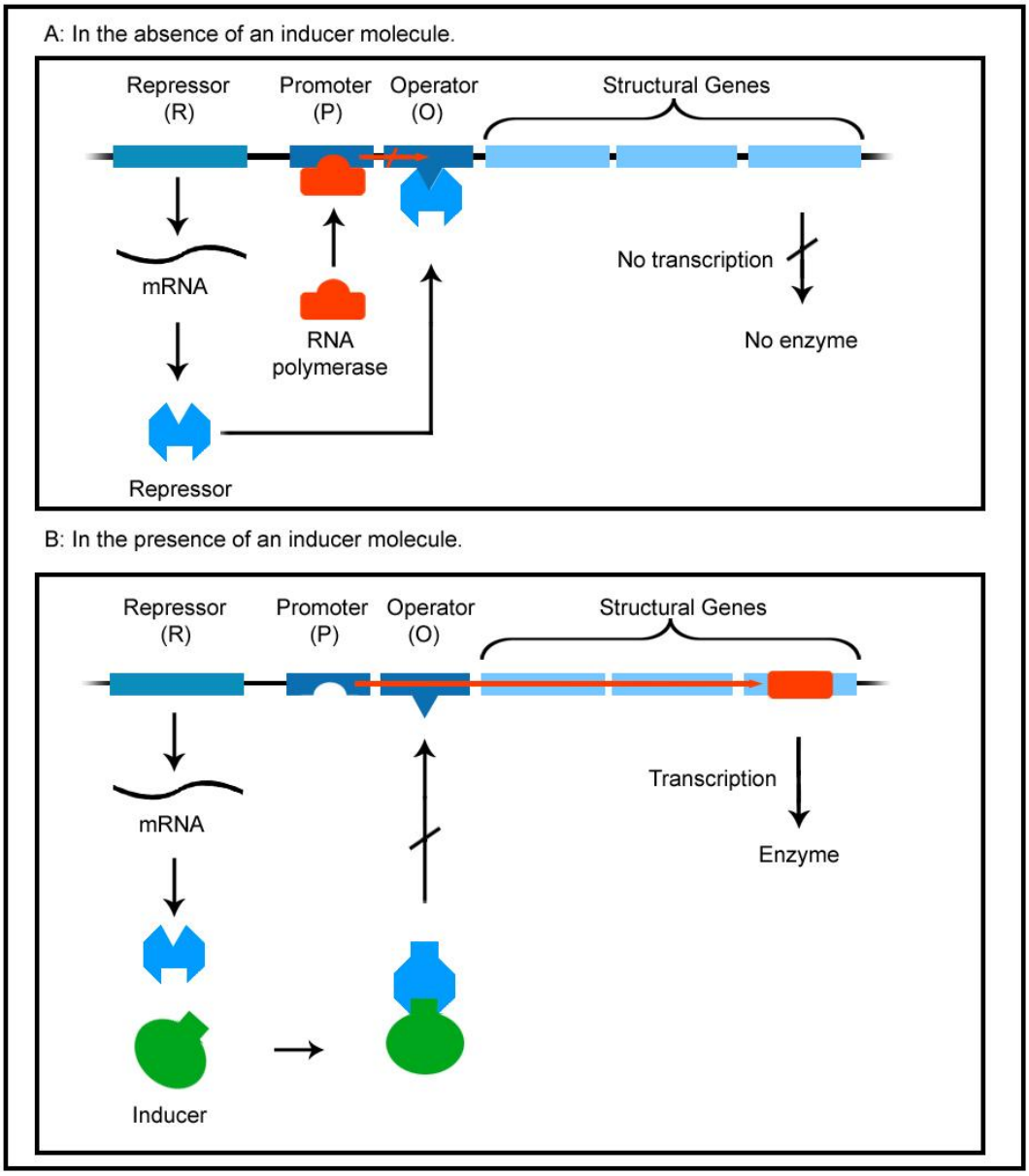

Figure 2.2: Illustration of lac operon and the effects in the absence of the substrate (Fig 2.2A) and in the presence of the substrate (Fig 2.2B).

MECHANISMS AFFECTING THE LEVEL OF INTRINSIC AND ACQUIRED DRUG RESISTANCE

2.1. Failure to activate the drug

Isoniazid (INH): INH is one of the most important first line anti-TB drugs. On entry into the cell by passive diffusion, INH is activated by the catalase-peroxidase enzyme (KatG) encoded by the *katG* gene to form isonicotinic acyl anions and other reactive radicals. It is speculated that the metabolic products that are generated by the activation of INH bind to and inactivate the protein encoded by the *inhA* gene which is an enoyl-acyl carrier protein (ACP) reductase, involved in mycolic acid synthesis (10). Inactivation of inhA leads to the inhibition of cell wall synthesis and cell death (75,138). Thus the MIC for INH is defined by the rate at which INH is activated and not by the interaction of the activated metabolic products with cellular processes (86). Accordingly, the INH MIC is largely determined by the rate of expression of *katG*. In contrast to *E. coli*, expression of the *katG* gene in members of the *M. tuberculosis* complex *(M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti)* is not regulated *oxyR* (114,115) as this gene is deleted in these members. Regulation of expression of the *katG* gene is thought to occur through alternative regulatory proteins present in the genome (42,43,103). Previous studies in non-mycobacterial species have suggested that expression of oxidative stress genes is coupled with iron metabolism via the ferric uptake regulator, Fur. This prompted scientists to investigate the relationship between the *fur* ortholoques in *M. tuberculosis* and *katG* expression. Whole genome sequencing of *M. tuberculosis* revealed that a *fur*-like gene, *furA,* is positioned 40 bp upstream from *katG* and forms a co-transcribed operon (43,81,86). Zhart and colleagues demonstrated that *katG* is over-expressed in a FurA knockout mutant leading to hypersensitivity to INH (decrease MIC) (133). This suggests that FurA acts as a repressor of *katG* expression (86,133). FurA negatively regulates the expression of the *furA* gene by binding to a region upstream of the *furA* gene (97). To date, only two *furA* mutant clinical isolates have been reported (86). However, these strains also had mutations in the *katG* gene and therefore it was speculated that the *furA* mutations may modulate the level of INH resistance.

Ethionamide (ETH): ETH is a structural analogue of INH which doesn't show cross resistance with INH and is thus used as a second-line drug for the treatment of MDR-TB and XDR-TB. ETH is a prodrug that

is activated by EthA (a FAD-containing monooxygenase enzyme), to generate metabolic by-products which exert a toxic effect upon the synthesis of the mycolic acid constituents of the mycobacterial cell wall (14,15,41,124). The expression of *ethA* is negatively regulated by its neighbouring gene, *ethR*, which encodes the protein EthR (15,41). EthR is a member of the TetR/CamR family of transcriptional regulators which binds cooperatively as a homo-octamer to the *ethA* operator (9), 5 to 16 nucleotides upstream from the *ethA* start codon (45). Thus, expression of *ethR* defines the level of expression of *ethA* which in turn determines the level the innate resistance to ETH (49). This is supported by the observation that over expression of *ethR* leads to repression of expression of *ethA* and to ETH resistance (15,41). Conversely, conditions leading to the down-regulation of *ethR* expresssion or mutation in *ethR* gene increases ETH susceptibility (lower MIC) (15). Similarly, the addition of compounds which bind to EthR (such as HexOc) and thereby prevent its binding to the *ethA* operator (50), promote expression of the *ethA* gene, continuous activation of ETH and increased ETH susceptibility (15,41).

2.2. Inactivation of drug

Aminoglycosides: Streptomycin (STR), Capreomycin (CAP) Amikacin (AM) and Kanamycin (KAN) are aminoglycosides used in the treatment of drug susceptible TB, MDR and XDR-TB. These compounds inhibit protein synthesis by binding to the ribosomes which are responsible for peptide elongation during translation (46,110). In *M. tuberculosis*, resistance to aminoglycosides develops through mutations in the *rrs* and *rpsl* genes encoding for the 16S rRNA and S12 ribosomal protein, respectively (88). In certain Mycobacteria (i.e. *M. fortuitum*, *M. chelonei*, *M. tuberculosis* and *M. smegmatis* (3,4,74)) aminoglycoside resistance also develops through the expression of acetyltransferases which transfer a functional group to the aminoglycoside structure, thereby preventing binding of the aminoglycoside to the ribosome (67,130). Three types of modifications have been demonstrated: O-phosphotransferases, O-nucleotidyltransferases and N-acetyltransferases (67). This modification prevents the binding of the aminoglycoside to the ribosome, the molecular target of the antibiotic (67,130). *N*-acetyltransferases are the most wide spread determinants of resistance to aminoglycosides (69). The *aac(2')-Id* gene confers resistance to aminoglycosides (4) and is expressed at low levels conferring low level innate aminoglycoside resistance. Up-regulation of expression of the *aac(2')-Id* gene by cloning the gene adjacent to a strong mycobacterial promoters resulted in higher aminoglycoside MIC values (74). This

suggests that the level of aminoglycoside resistance depends on the strength of the promoter responsible for the transcription of the *aac(2')-lb* (74).

The *aph(3")-lc* gene, encoding for a 3"-O-phosphotransferase, has been shown to confer resistance to streptomycin in *M. fortuitum*, however, no homologues of this gene could be found in other Mycobacterium species (93). Also no additional information is available on the transcriptional regulation for this gene in *M. fortuitum*. However in *Pseudomonas aeruginosa* the *aph(3')-llb* gene (encoding for an aminoglycoside-phosotransferase gene) is under the positive control of a surrogate regulator HpaA (135). Therefore the transcriptional regulation for *aph(3")-lc* needs to be investigated further in mycobacteria.

Isoniazid: INH is inactivated when an acetyl group is transferred to the free amino group to form an acetylamide (84). This reaction is catalyzed by the human N-Acetyl transferase (NAT2) (105), as well as by the *M. tuberculosis* NAT enzyme (122). Heterologous expression of *M. tuberculosis* NAT in *M. smegmatis* resulted in a three-fold increase in INH resistance (84) while increased sensitivity to INH was observed in a *M. smegmatis nat* knockout mutant (85). Both of the above studies speculated that NAT might be involved in innate INH resistance. However, this is controversial as a recent study has demonstrated through kinetic characterization that INH is a poor substrate for the NAT enzyme (107).

Rifampicin (RIF): RIF binds to the beta subunit of the RNA polymerase, thereby hindering transcription (119). Single nucleotide substitutions in the 81 bp core region of the *rpoβ* gene (also known as the RIF resistance determining region; RRDR) are associated with RIF resistance altering the structural conformational of the RNA polymerase thereby decreasing the binding affinity between RIF and the RNA polymerase (120). Interestingly, *M. smegmatis* demonstrates RIF resistance in the absence of *rpoB* mutations (53). This suggests that other mechanisms are involved in RIF resistance. Many fast growing mycobacterium strains, including *M. smegmatis, M chelonae, M. flavescens, M. vaccae* and *M. parafortuitum*, have been reported to inactivate RIF by ADP-ribosylation (118). It has been shown that RIF ribosylation is a major contributor to low level RIF resistance in *M. smegmatis* (87) and is encoded by the *arr-ms* gene. The ADP-ribosyl transferases transfer an ADP-ribose unit to a susceptible amino acid residue on the target protein with the loss of a nicotinamide molecule (16). Significantly, this genes is absent from the *M. tuberculosis* genome thereby explaining RIF susceptibility in pan susceptible isolates.

2.3. Alteration of drug target's activity

It is well documented that acquired drug resistance in mycobacteria develops through spontaneously chromosomal mutations in the gene encoding for the drug target (59). It is now known that different levels of drug resistance can arise through mutations at different positions in the target gene (132) or mutations in other non-target genes (123).

Isoniazid: Mutations at different positions in the *inhA* gene, resulting in structural changes in InhA protein have been reported to confer isoniazid resistance, however these mutations are rarely seen in clinical isolates (88). Mutations in this gene generally confers low level resistance (<0.5 μg/ml) (1,90). Many of these low level resistance clinical isolates do not have mutations in the *katG* gene (1). Mutations in the *katG* gene encoding for catalase perozidase responsible for the activation of INH, also confers resistance to INH. These mutations result in high level resistance to INH and occur in 30-60% of all INH resistant isolates (1,88).

Rifampicin: RIF binds to the beta subunit of the RNA polymerase, thereby hindering transcription (119). Single nucleotide substitutions in the 81 bp core region of the *rpoβ* gene (also known as the RIF resistance determining region; RRDR) are associated with RIF resistance (REF) by altering the structural conformational of the RNA polymerase thereby decreasing the binding affinity between RIF and the RNA polymerase. The level of RIF resistance in *M. tuberculosis* is dependent on the position of the mutation in the *rpoB* gene, however, most *rpoB* mutations cause resistance above the critical concentration of 2 μg/ml. The MIC for RIF has been reported to range from 32 to 256 on 7H10 solid media (56) (Table 2.2). In clinical isolates mutations in codon Ser531 and His526 account for more than 75% of RIF resistance. The frequency at which these mutations occur is thought to reflect the fitness cost incurred by the respective mutation (22,70).

Ethambutol (EMB): EMB is a first line anti-TB drug which inhibits the synthesis of cell wall arabinan, a component of the cell wall structural molecule, arabinogalactan. EMB binds to arbinosyltransferases which is encoded by the *emb* genes (17,116). The inhibition of arabinan synthesis leads to accumulation

of mycolic acids and eventually to cell death (89). Mutations at codon 306 in the embB gene was usually associated with high level EMB resistance (113). However, recently it has been shown that Met306Leu substitutions are associated with high level EMB resistance, while Met306Ile substitutions are associated with low level EMB resistance (57,111).

Streptomycin (STR): STR is an alternative first line drug used in the treatment of TB. In *M. Tuberculosis* the effect of STR has been demonstrated to take place at ribosomal level where it interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and r*psL*) (109). This results in the induction of ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis (55). Mutations associated with STR resistance have been identified in the *rrs* and *rpsL* genes. In addition it has been shown that one-third of STR resistant clinical isolates nucleotide changes in these genes, suggesting that other mechanisms for STR resistance exist in *M. Tuberculosis* (48,79). It has been shown that mutations in the rpsL gene are associated with high level resistance, while mutations in the rrs gene are associated with intermediate level of resistance (73). Isolates with a wild-type *rpsL* and *rrs* genotype exhibited a low-level resistance phenotype (73).

Ofloxacin (OFX): OFX is a FQ used as a second line drugs for the treatment of MDR-TB. In *M. tuberculosis* it targets and inactivates a type II DNA polymerase, DNA gyrase (44) (117). Mutations in the genes encoding for the DNA gyrase, *gyrA* and *gyrB*, are responsible for conferring resistance to FQ by introducing negative supercoils in the circular DNA molecules (88). Asp94Gly substitutions in the gyrA gene are associated with high level OFX resistance (61). It is speculated that this specific mutation render the DNA gyrased conformation more difficult for FQ's to bind. This would result in higher MIC values for this drug.

2.4. Differential expression of drug target

Isoniazid: Mutations in the promoter region of *inhA* gene are more frequently seen in clinical isolates. These mutations lead to the over-expression of the *inhA* gene resulting in an increase in the concentration if InhA which partially overcomes the toxic metabolic byproducts generated by KatG leading to low level INH (62,68).

Ethambutol: EMB resitance is further example where transcriptional regulation of the drug target would affect the level of drug resistance (REF). The *embCAB*-operon which encodes the target genes EmbA, EmbB and EmbC is found in most mycobacterial species. EmbA and EmbB are enzymes that catalyse the arabinosylation of arabinogalactan, while EmbC synthesises lipoarabinomannan (47,136). In *M. avium* the *emb* gene cluster contains only the *embAB* genes and an additional putative transcriptional regulator, *embR*, immediately upstream of the *embAB* genes (17,89). EmbR is a multidomain protein and possesses a DNA binding winged helix-turn-helix domain, a bacterial transcription activation domain and a forkhead-associated (FHA) domain (101). The three dimensional structure of EmbR suggests that it acts as a transcriptional regulator (6). It was observed that EmbR acts as a transcriptional regulator by modulating the arabinosyltransferase activity *in vitro* (17). In the *M. tuberculosis* and *M. smegmatis* genome, an *embR* homolog is located 2 MB from the *embCAB* locus, leading to the hypothesis that this homolog may also modulate the level of arbinosyltransferase activity (34,89).

The Ser/Thr protein kinase, PnkH is another protein involved in transcriptional regulation of *embCAB* (76). This Ser/Thr protein kinase is located in the mycobacterial membrane and therefore it is speculated that PnkH is autophosphorylated on sensing external stimuli. This significantly increases the protein kinase activity (76). In turn PnkH phosphorylates the FHA domain of EmbR, which enhances the binding activity towards the promoter regions of *embCAB* (76,101) thereby enhancing transcription leading to a higher concentration of EmbB, EmbA and EmbC, influencing the lipoarabinomannan:lipomannan ratio in the cell. Similarly, Mutations in the EmbR FHA domain were previously shown to be associated with EMB resistance (89).

Since EMB resistance depends on the gene copy number of EmbB and EmbA, increased expression of *embCAB* through increased activation by the PnkH-EmbR pair would be predicted to result in increased levels EMB resistance (17,101).

2.5. Increased efflux of drug

Low-level intrinsic resistance in the absence of mutations in the known drug resistance causing genes may be due to either permeability or active efflux. Thus, the intracellular concentration of a given drug will depend on the balance between its influx and efflux (126). This balance may be disturbed by treatment as antibiotics can serve as inducers, regulating the expression of efflux pumps at the level of gene transcription by interacting with regulatory systems (37). Bacterial drug efflux pumps are generally classified on the basis of their energy source (64). The ATP binding cassette (ABC) superfamily are considered as primary transporters and make use of ATP as an energy source (19,23,37,82). The Major Facilitator Superfamily (MFS) (37,106,108), Small Multidrug Resistance (SMR) family (65), Resistance-Nodulation-cell Division (RND) family (34,82) and Multidrug And Toxic compounds Extrusion family (MATE) are secondary transporters which are driven by proton (H^+) influx (37).

In Mycobacteria the following efflux pumps involved in drug resistance have been identified and described.

MFS efflux pumps

To date, six MFS efflux pumps have been described in Mycobacteria that may define the level of intrinsic resistance to various anti-TB drugs. The Rv1634 efflux pump in *M. tuberculosis* conferred resistance to FQ (ciprofloxacin, norfloxacin, ofloxacin, lomefloxacin) when over expressed in *M. smegmatis* (38).

The Tet(V) efflux pump, isolated from *M. smegmatis*, was shown to increase the minimum inhibitory concentration (MIC) of tetracycline when over expressed (40). However, this efflux pump has only been identified in *M. smegmatis* and *M. fortuitum* (40).

The Tap efflux pump from *M. fortuitium* increased resistance to aminoglycosides when expressed in *M. smegmatis* (2). The activity of Tap efflux pumps in *M. fortunium* can be inhibited by several efflux pump inhibitors such as reserpine and CCCP, leading to lower levels of resistance or increased susceptibility (91). Its homologue, Rv1258c, in *M. tuberculosis* conferred resistance only to tetracycline (2). However, analysis of Rv1258c gene expression of a clinical *M. tuberculosis* isolate exposed to RIF and ofloxocin showed increased transcript levels (106). Although RIF and OFX were not found to be substrates of Tap (2), Sidiqi *et al* suggested that the high level resistance to rifampicin could by explained by the over expression of Rv1258c (106). This suggests that certain efflux pumps may be induced by exposure to drugs.

The P55 efflux pump in *M. bovis* and its *M. tuberculosis* homologue, *Rv1410c*, also confers resistance to aminoglycosides and tetracycline (20) (108). Expressing P55 in *M. smegmatis* resulted in an 8-fold increase in the MIC for STR (108). This efflux pump could explain STR resistance in STR-resistant *M. tuberculosis* isolates that do not harbour mutations in the *rrs* and *rpsL* genes (88). *Rv1410c* is organized in an operon with *lprG* which encodes for a membrane protein, P27 (20). Although the function of this gene product is unknown, disruption of *lprG* abrogates the expression of *Rv1410c* leading to strong attenuation of virulence in mice (21). This suggested that P27 may have a direct or indirect regulatory role in P55 expression. Recently it has been shown that the deletion of P55 in *M. Bovis* BCG resulted in increased susceptibility to a range of toxic compounds including RIF and clofazimine (92).

Expression of the *M. tuberculosis* H37Rv *epfA* gene in *M. smegmatis* increased in response to INH treatment (129). *EpfA* encodes for a putative efflux protein (EfpA) with a similar secondary structure to that of members of a transporter family known for the mediation of antibiotic resistance in bacteria and yeast (QacA transporter family) (37). Although no association between the EfpA and drug resistance could be made, the deletion of the *epfA* homologue in *M. smegmatis* increased the susceptibility of the isolates to ethidium bromide, gentamicin, FQ and acriflavine (37).

Limited experimental data exists to provide evidence that the regulation of efflux genes influence the level of drug resistance in *M. tuberculosis*. However, functional analysis has been done extensively on the MFS LrfA efflux pump (117). The *lfrA* gene in *M. smegmatis* encodes a transporter which confers low-level resistance to fluoroquinolones, acriflavine and ethidiumbromide when over expressed (66,117). Disruption of *lfrA* resulted in increased susceptibility to FQ, acriflavine and ethidiumbromide (65,98). Regulatory mutations leading to constitutive expression and induction by the substrates of the pump may lead to increased expression of lfrA (37).

An open reading frame (570 bp), *lfrR*, upstream of the *lfrA* gene with homology to several TetR transcriptional proteins was previously identified (65). TetR transcriptional regulators are characterized by a conserved DNA binding domain (helix-turn-helix at the N-terminal) and a ligand binding domain (C-terminal region). Binding of an inducing ligand to the C-terminal region results in conformational changes in the N-terminal region, reducing its affinity to its target promoter DNA (54). It was shown that a 390 base pare deletion of *lfrR* resulted in increased CIP and norfloxacin resistance and increased *lfrA* expression (65). This suggested that LfrR acts as a repressor that negatively regulates the production of LfrA. Realtime (RT)-PCR experiments revealed that the *lfrR* and *lfrA* genes are organized as an operon, with a promoter 220 base pares upstream from *lfrR* (27). LfrR represses lfrA expression by binding directly to the promoter region of lfR-lfA (27).

Acriflavine acts as a ligand inducer for LfrR (27). It induces a conformational change in this repressor, leading to a reduction in its DNA binding affinity, which may lead to increased expression of *lfrA* (27). Although ciprofloxin acts as a substrate of *lfrA* it showed no interaction with LfrR, suggesting that not all substrates of *lfrA* act as inducers (27). The combination of mutations *gyrA* and *gyrB* genes (72) and a FQ efflux pump may lead to different (increased) levels of drug resistance. As there is no known homology of *lfrA* in *M. tuberculosis* it is speculated that the regulation of efflux pumps may be involved in resistance to FQ in *M. tuberculosis* (37,38).

An additional 16 putative MFS drug efflux pumps have been identified in *M. tuberculosis* through whole genome analysis and comparative bioinformatics (38), however, their role in intrinsic drug resistance remains to be determined.

SMR family drug transporters

Only one member of the SMR family of drug transporters for mycobacterium has been described thus far. The *mmr*-like gene (encoding for the protein Mmr) are present in *M. tuberculosis* (*Rv3065*), *M. simiae, M. gordonae, M. marinum, M. smegmatis* and *M. bovis (39)*. When the *mmr* gene from *M. tuberculosis* was expressed in *M. smegmatis*, it conferred resistance to a number of toxins and drugs such as tetraphenyl phosphonium, ethidiumbromide, erythromycin, safranin O and pyronin Y (39). Furthermore, the deletion of the *mmr* homologue in *M. smegmatis* increases the susceptibility of the bacterium to cationic dyes and FQ, suggesting that this protein plays an important role in the intrinsic resistance of *M. smegmatis* (65).

RND drug transporters

Sequencing and analysis of the whole genome sequence of *M. tuberculosis* revealed the presence of 13 putative transmembrane proteins which are members of the RND family of drug transporters (34). The MmpL (mycobacterial membrane proteins, large) proteins are confined to mycobacteria (83). Over expression of the *M. tuberculosis mmpL7* gene *M. smegmatis* increase resistance to INH to a level 32 times higher that the MIC of the wild type (83), suggesting that MmpL plays a role in the detoxification process by which *M. tuberculosis* limits the effects of INH.

ABC drug transporters

A total of 37 ABC transporter genes have been identified in the genome *M. tuberculosis* (23). The ABC transporters are characterised by at least 4 functional domains (two membrane-spanning domains and two

nucleotide-binding domains), however, only a few have been shown to be associated with drug resistance in *mycobacteria*. Two operons containing ABC transporter genes, doxorubicin-resistance operon (*drAB*) (34) and the *Rv2686c-Rv2687c-Rv2688c* operon, have been described for *M. tuberculosis*. Whole genome sequencing of *M. tuberculosis*, revealed the presence of the doxorubicin-resistance operon, *drrAB,* which encodes for an ABC drug transporter (34). DrrAB confers resistance to antibiotics such as tetracycline, erythromycin, EMB, norfloxacin and STR when expressed in *M. smegmatis.* The resistant phenotype could be reversed by efflux pump inhibitors such as verapamil and reserpine (30). The *Rv2686c-Rv2687c-Rv2688c* operon encodes for an ABC transporter responsible for FQ efflux (82). The pump confers resistance to CIP (8 x MIC) and norfloxacin (2 x MIC) when over expressed in *M. smegmatis* (82).

The phosphate specific transporter (Pst) is involved in phosphate transport and has been reported in numerous bacteria, including *M. tub*erculosis (12,24). PstB overexpression was observed in an in vitro generated CIP mutant (11). Efflux pump inhibitors, reserpine and verapamil, were shown to reverse the resistance phenotype, suggesting that PstB plays a role in increasing CIP resistance levels (11). Sensitivity to CIP, OFL and sparfloxacin was observed with the disruption of the *pst operon* (19).

2.6. Drug tolerance

Little is known about the functional roles of *M. tuberculosis* genes which are up regulated in response to an antibiotic (33). It is hypothesized that such transcriptional changes might induce antimicrobial tolerance and thereby defining the level of intrinsic drug resistance. An example of antibiotic-induced genes in *M. tuberculosis* is the *iniBAC* operon which is induced by INH and EMB (7,8). Over expression of *M. tuberculosis iniA* gene in *M. bovis* BCG upon exposure to INH and EMB resulted in a tolerance-like phenotype to these antibiotics (32). It is suggested that IniA preserves cellular functions normally disrupted by these antibiotics (32). Deletions of *iniA* in *M. tuberculosis* increases its susceptibly to INH (32).

In addition, it has been shown that the *lsr2* gene product down regulates the transcription of the *iniBAC* genes in *M. tuberculosis (33)).* Lsr2 is a small histone-like protein that directly interacts with DNA, forming large oligomeric complexes through DNA bridging (29,33). Deletion of *lsr2* in *M. smegmatis* resulted in increased resistance to EMB (33). Thus a mutation or reduced expression in the *lsr2* gene,

leading to decreased DNA binding affinity might result in increased expression of *iniBAC* and resistance to INH and EMB.

The *M. tuberculosis* genome contains 7 *whiB*-like genes (*whiB*1-7) which encode for putative transcriptional regulators (34,80). Geiman *et al* showed that INH, ethambutol and cycloserine stimulated *whiB2* transcription. Aminoglycosides such as STR and kanamycin induced the transcription of *whiB7* (51). In *Streptomyces lividans* and *S. coelicolor whiB2* is responsible for multi-drug resistance and associated with higher levels of drug resistance (18,78). Thus it was suggested that whiB7 might play a similar role in the drug resistance of *M. tuberculosis* (78). Exposure of *M. tuberculosis* to STR led to the increased expression of *whiB7* (78). Microarray analysis revealed that upon tetracycline exposure the expression of 12 other genes was temporally dependant on the initial induction of *whiB7* (78). This suggests that WhiB7 acts as a regulator, activating a regulon involved in intrinsic antibiotic resistance (78).

CONCLUDING REMARKS

It is now known that drug resistance in Mycobacteria is influenced by mechanisms other than the classical drug resistant gene causing mutations. This review discussed various mechanisms by which Mycobacteria have evolved to elevate its level of resistance to certain drugs. Varying levels of resistance are contributed by acient intrinsic mechanisms such as the rigid mycobacteria cell wall and active efflux pumps. Mycobacteria also has aquired novel mechanisms through chromosomal alterations in non-classical drug resitance genes and regulatory units. The combination of these mechanisms would result in strains which become hyper resistant to anti-TB drugs. This demonstrates the complexicity of drug resistance in Mycobacteria. The indentification of novel mehanisms that regulate the level of drug resistance can serve as potential candidates for future drug design to improve the treatment of TB.

Table 2.1: Summary of genes associated with drug resistance in *M. tubercolosis*.

	Drug		**Drug target**	**Mutations**	**Enzyme**	**Reference**
First line drugs	Isoniazid (INH)		InhA	*katG*	Catalase peroxidase	*(31,88,90,94)*
				ahpC	Alkyl hydroperoxide	
				inhA	fatty acid enoyl acyl carrier protein reductase A	
				kasA	β-ketoacyl-ACP	
				ndh	NADH dehydrogenease	
	Rifampicin (RIF)		β subunit RNA polymerase	*rpoB*	β subunit RNA polymerase	*(28,119)*
	Pyrazinamide (PZA)		No specific target	*pncA*	Pyrazinomidase	*(100,137)*
	Ethambutol (EMB)		*embB*	*embCAB*	arabinosyl transferase	*(88,95,121)*
Second line drugs	**Aminoglycosides**	Streptomycin (STR)	16S rRNA ribosomal subunits	*rpsl*	16S rRNA ribosomal subunits	*(5,71,95)*
				rrs		
		Capreomycin (CAP)		*tlyA*		
		Kanamycin (KAN)				
		Aminokacin (AMI)				
	Ethionamide (ETH)		InhA	*inhA*	fatty acid enoyl acyl carrier protein reductase A	*(45,77)*

			ethA	flavin monooxygenase	
			ethR	Transcriptional regulator	
	Fluoroquinolones (FQ)	DNA gyrase	*gyrA*	DNA gyrase	*(44)*
			gyrB		

Table 2.2: Level of RIF resistance in clinical isolates and in vitro selected mutants.

	Codon	**MIC (µl/ml)**	**Reference**
Clinical	His526Tyr	10-64	(112)
		512	(35)
	Ser531Leu	> 64	(112)
		>512	(35)
In Vitro (selected mutants)	Ser522Leu	> 16	(70)
		8-16	(56)
	His526Tyr	> 32	(70)
		≥32 - ≥256	(56)
	His531Trp/Leu	> 32	(70)
		≥ 32 - ≥ 256	(56)

Table 2.3: Transcriptional regulators of the molecular mechanisms of drug resistance in mycobacteria.

	Regulator gene	**Regulator protein**	**Target genes of regulator**	**Drug resistance effected**	**References**
Activator	*embR*	EmbR	*EmbCAB*	Ethambutol	(17,89,101)
	whiB7	WhiB7	Regulon		(78)
Repressor	*ethR*	EthR	*ethA*	Ethionamide	(45,78)
	furA	Fur A	*katG*	Isoniazid	(86,133)
	lfrR	LfrR	*lfrA*	Fluoriquinolones	(66,98)
	lsr2	Lsr2	*iniBAC*	Isoniazid Ethambutol	(32,33,89,90)

REFERENCES

1. **Abe, C., I. Kobayashi, S. Mitarai, M. Wada, Y. Kawabe, T. Takashima, K. Suzuki, L. H. Sng, S. Wang, H. H. Htay, and H. Ogata**. 2008. Biological and molecular characteristics of Mycobacterium tuberculosis clinical isolates with low-level resistance to isoniazid in Japan. J.Clin.Microbiol. **46**:2263-2268.

2. **Ainsa, J. A., M. C. Blokpoel, I. Otal, D. B. Young, K. A. De Smet, and C. Martin**. 1998. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in Mycobacterium fortuitum and Mycobacterium tuberculosis. J.Bacteriol. **180**:5836-5843.

3. **Ainsa, J. A., C. Martin, B. Gicquel, and R. Gomez-Lus**. 1996. Characterization of the chromosomal aminoglycoside 2'-N-acetyltransferase gene from Mycobacterium fortuitum. Antimicrob.Agents Chemother. **40**:2350-2355.

4. **Ainsa, J. A., E. Perez, V. Pelicic, F. X. Berthet, B. Gicquel, and C. Martin**. 1997. Aminoglycoside 2'-N-acetyltransferase genes are universally present in mycobacteria: characterization of the aac(2')-Ic gene from Mycobacterium tuberculosis and the aac(2')-Id gene from Mycobacterium smegmatis. Mol.Microbiol. **24**:431-441.

5. **Alangaden, G. J., B. N. Kreiswirth, A. Aouad, M. Khetarpal, F. R. Igno, S. L. Moghazeh, E. K. Manavathu, and S. A. Lerner**. 1998. Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **42**:1295-1297.

6. **Alderwick, L. J., V. Molle, L. Kremer, A. J. Cozzone, T. R. Dafforn, G. S. Besra, and K. Futterer**. 2006. Molecular structure of EmbR, a response element of Ser/Thr kinase signaling in Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A .

7. **Alland, D., I. Kramnik, T. R. Weisbrod, L. Otsubo, R. Cerny, L. P. Miller, W. R. Jacobs, Jr., and B. R. Bloom**. 1998. Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A **95**:13227-13232.

8. **Alland, D., A. J. Steyn, T. Weisbrod, K. Aldrich, and W. R. Jacobs, Jr.** 2000. Characterization of the Mycobacterium tuberculosis iniBAC promoter, a promoter that responds to cell wall biosynthesis inhibition. J.Bacteriol. **182**:1802-1811.

9. **Aramaki, H., N. Yagi, and M. Suzuki**. 1995. Residues important for the function of a multihelical DNA binding domain in the new transcription factor family of Cam and Tet repressors. Protein Eng **8**:1259-1266.

10. **Banerjee, A., E. Dubnau, A. Quemard, V. Balasubramanian, K. S. Um, T. Wilson, D. Collins, G. de Lisle, and W. R. Jacobs, Jr.** 1994. inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science **263**:227-230.

11. **Banerjee, S. K., K. Bhatt, S. Rana, P. Misra, and P. K. Chakraborti**. 1996. Involvement of an efflux system in mediating high level of fluoroquinolone resistance in Mycobacterium smegmatis. Biochem.Biophys.Res.Commun. **226**:362-368.

12. **Banerjee, S. K., P. Misra, K. Bhatt, S. C. Mande, and P. K. Chakraborti**. 1998. Identification of an ABC transporter gene that exhibits mRNA level overexpression in fluoroquinolone-resistant Mycobacterium smegmatis. FEBS Lett. **425**:151-156.

13. **Barlow, M.** 2009. What antimicrobial resistance has taught us about horizontal gene transfer. Methods Mol.Biol. **532**:397-411.

14. **Barry, C. E., III, R. E. Lee, K. Mdluli, A. E. Sampson, B. G. Schroeder, R. A. Slayden, and Y. Yuan**. 1998. Mycolic acids: structure, biosynthesis and physiological functions. Prog.Lipid Res. **37**:143-179.

15. **Baulard, A. R., J. C. Betts, J. Engohang-Ndong, S. Quan, R. A. McAdam, P. J. Brennan, C. Locht, and G. S. Besra**. 2000. Activation of the pro-drug ethionamide is regulated in mycobacteria. J.Biol.Chem. **275**:28326-28331.

16. **Baysarowich, J., K. Koteva, D. W. Hughes, L. Ejim, E. Griffiths, K. Zhang, M. Junop, and G. D. Wright**. 2008. Rifamycin antibiotic resistance by ADP-ribosylation: Structure and diversity of Arr. Proc.Natl.Acad.Sci.U.S.A **105**:4886-4891.

17. **Belanger, A. E., G. S. Besra, M. E. Ford, K. Mikusova, J. T. Belisle, P. J. Brennan, and J. M. Inamine**. 1996. The embAB genes of Mycobacterium avium encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc.Natl.Acad.Sci.U.S.A **93**:11919-11924.

18. **Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood**. 2002. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature **417**:141-147.

19. **Bhatt, K., S. K. Banerjee, and P. K. Chakraborti**. 2000. Evidence that phosphate specific transporter is amplified in a fluoroquinolone resistant Mycobacterium smegmatis. Eur.J.Biochem. **267**:4028-4032.

20. **Bigi, F., A. Alito, M. I. Romano, M. Zumarraga, K. Caimi, and A. Cataldi**. 2000. The gene encoding P27 lipoprotein and a putative antibiotic-resistance gene form an operon in Mycobacterium tuberculosis and Mycobacterium bovis. Microbiology **146 (Pt 4)**:1011-1018.

21. **Bigi, F., A. Gioffre, L. Klepp, S. M. de la Paz, A. Alito, K. Caimi, V. Meikle, M. Zumarraga, O. Taboga, M. I. Romano, and A. Cataldi**. 2004. The knockout of the lprG-Rv1410 operon produces strong attenuation of Mycobacterium tuberculosis. Microbes.Infect. **6**:182-187.

22. **Billington, O. J., T. D. McHugh, and S. H. Gillespie**. 1999. Physiological cost of rifampin resistance induced in vitro in Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **43**:1866-1869.

23. **Braibant, M., P. Gilot, and J. Content**. 2000. The ATP binding cassette (ABC) transport systems of Mycobacterium tuberculosis. FEMS Microbiol.Rev. **24**:449-467.

24. **Braibant, M., P. Lefevre, W. L. de, P. Peirs, J. Ooms, K. Huygen, A. B. Andersen, and J. Content**. 1996. A Mycobacterium tuberculosis gene cluster encoding proteins of a phosphate transporter homologous to the Escherichia coli Pst system. Gene **176**:171-176.

25. **Brantl, S.** 2004. Bacterial gene regulation: from transcription attenuation to riboswitches and ribozymes. Trends Microbiol. **12**:473-475.

26. **Brennan, P. J. and H. Nikaido**. 1995. The envelope of mycobacteria. Annu.Rev.Biochem. **64**:29-63.

27. **Buroni, S., G. Manina, P. Guglierame, M. R. Pasca, G. Riccardi, and R. E. De**. 2006. LfrR is a repressor that regulates expression of the efflux pump LfrA in Mycobacterium smegmatis. Antimicrob.Agents Chemother. **50**:4044-4052.

28. **Chaves, F., M. Alonso-Sanz, M. J. Rebollo, J. C. Tercero, M. S. Jimenez, and A. R. Noriega**. 2000. rpoB mutations as an epidemiologic marker in rifampin-resistant Mycobacterium tuberculosis [In Process Citation]. Int.J.Tuberc.Lung Dis. **4**:765-770.

29. **Chen, J. M., H. Ren, J. E. Shaw, Y. J. Wang, M. Li, A. S. Leung, V. Tran, N. M. Berbenetz, D. Kocincova, C. M. Yip, J. M. Reyrat, and J. Liu**. 2008. Lsr2 of Mycobacterium tuberculosis is a DNA-bridging protein. Nucleic Acids Res. **36**:2123-2135.

30. **Choudhuri, B. S., S. Bhakta, R. Barik, J. Basu, M. Kundu, and P. Chakrabarti**. 2002. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes drrA and drrB of Mycobacterium tuberculosis. Biochem.J. **367**:279-285.

31. **Clemente, W. T., S. S. Soares Lima, M. Palaci, M. S. Silva, R. Sumnienski, V, E. R. la Costa, L. Possuelo, P. I. Cafrune, F. K. Ribeiro, H. M. Gomes, and J. C. Serufo**. 2008. Phenotypic and genotypic characterization of drug-resistant Mycobacterium tuberculosis strains. Diagn.Microbiol.Infect.Dis. **62**:199-204.

32. **Colangeli, R., D. Helb, S. Sridharan, J. Sun, M. Varma-Basil, M. H. Hazbon, R. Harbacheuski, N. J. Megjugorac, W. R. Jacobs, Jr., A. Holzenburg, J. C. Sacchettini, and D. Alland**. 2005. The Mycobacterium tuberculosis iniA gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. Mol.Microbiol. **55**:1829-1840.

33. **Colangeli, R., D. Helb, C. Vilcheze, M. H. Hazbon, C. G. Lee, H. Safi, B. Sayers, I. Sardone, M. B. Jones, R. D. Fleischmann, S. N. Peterson, W. R. Jacobs, Jr., and D. Alland**. 2007. Transcriptional regulation of multi-drug tolerance and antibiotic-induced responses by the histone-like protein Lsr2 in M. tuberculosis. PLoS.Pathog. **3**:e87.

34. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and a. et**. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393**:537-544.

35. **Cummings, M. P. and M. R. Segal**. 2004. Few amino acid positions in rpoB are associated with most of the rifampin resistance in Mycobacterium tuberculosis. BMC.Bioinformatics. **5**:137.

36. **Danilchanka, O., C. Mailaender, and M. Niederweis**. 2008. Identification of a novel multidrug efflux pump of Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **52**:2503-2511.

37. **De Rossi, E., J. A. Ainsa, and G. Riccardi**. 2006. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol.Rev. **30**:36-52.

38. **De Rossi, E., P. Arrigo, M. Bellinzoni, P. A. Silva, C. Martin, J. A. Ainsa, P. Guglierame, and G. Riccardi**. 2002. The Multidrug Transporters Belonging to Major Facilitator Superfamily in Mycobacterium tuberculosis. Mol.Med. **8**:714-724.

39. **De Rossi, E., M. Branzoni, R. Cantoni, A. Milano, G. Riccardi, and O. Ciferri**. 1998. mmr, a Mycobacterium tuberculosis gene conferring resistance to small cationic dyes and inhibitors. J.Bacteriol. **180**:6068-6071.

40. **De, R. E., M. C. Blokpoel, R. Cantoni, M. Branzoni, G. Riccardi, D. B. Young, K. A. De Smet, and O. Ciferri**. 1998. Molecular cloning and functional analysis of a novel tetracycline resistance determinant, tet(V), from Mycobacterium smegmatis. Antimicrob.Agents Chemother. **42**:1931-1937.

41. **DeBarber, A. E., K. Mdluli, M. Bosman, L. G. Bekker, and C. E. Barry, III**. 2000. Ethionamide activation and sensitivity in multidrug-resistant Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A **97**:9677-9682.

42. **Deretic, V., W. Philipp, S. Dhandayuthapani, M. H. Mudd, R. Curcic, T. Garbe, B. Heym, L. E. Via, and S. T. Cole**. 1995. Mycobacterium tuberculosis is a natural mutant with an

inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. Mol.Microbiol. **17**:889-900.

43. **Deretic, V., J. Song, and E. Pagan-Ramos**. 1997. Loss of oxyR in Mycobacterium tuberculosis. Trends Microbiol. **5**:367-372.

44. **Drlica, K. and M. Malik**. 2003. Fluoroquinolones: action and resistance. Curr.Top.Med.Chem. **3**:249-282.

45. **Engohang-Ndong, J., D. Baillat, M. Aumercier, F. Bellefontaine, G. S. Besra, C. Locht, and A. R. Baulard**. 2004. EthR, a repressor of the TetR/CamR family implicated in ethionamide resistance in mycobacteria, octamerizes cooperatively on its operator. Mol.Microbiol. **51**:175-188.

46. **Escalante, P., S. Ramaswamy, H. Sanabria, H. Soini, X. Pan, O. Valiente-Castillo, and J. M. Musser**. 1998. Genotypic characterization of drug-resistant Mycobacterium tuberculosis isolates from Peru. Tuber.Lung Dis. **79**:111-118.

47. **Escuyer, V. E., M. A. Lety, J. B. Torrelles, K. H. Khoo, J. B. Tang, C. D. Rithner, C. Frehel, M. R. McNeil, P. J. Brennan, and D. Chatterjee**. 2001. The role of the embA and embB gene products in the biosynthesis of the terminal hexaarabinofuranosyl motif of Mycobacterium smegmatis arabinogalactan. J.Biol.Chem. **276**:48854-48862.

48. **Finken, M., P. Kirschner, A. Meier, A. Wrede, and E. C. Bottger**. 1993. Molecular basis of streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol.Microbiol. **9**:1239-1246.

49. **Frenois, F., A. R. Baulard, and V. Villeret**. 2006. Insights into mechanisms of induction and ligands recognition in the transcriptional repressor EthR from Mycobacterium tuberculosis. Tuberculosis.(Edinb.) **86**:110-114.

50. **Frenois, F., J. Engohang-Ndong, C. Locht, A. R. Baulard, and V. Villeret**. 2004. Structure of EthR in a ligand bound conformation reveals therapeutic perspectives against tuberculosis. Mol.Cell **16**:301-307.

51. **Geiman, D. E., T. R. Raghunand, N. Agarwal, and W. R. Bishai**. 2006. Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven Mycobacterium tuberculosis whiB-like genes. Antimicrob.Agents Chemother. **50**:2836-2841.

52. **Gupta, R. and M. Espinal**. 2003. A prioritised research agenda for DOTS-Plus for multidrug-resistant tuberculosis (MDR-TB). Int.J.Tuberc.Lung Dis. **7**:410-414.

53. **Hetherington, S. V., A. S. Watson, and C. C. Patrick**. 1995. Sequence and analysis of the rpoB gene of Mycobacterium smegmatis. Antimicrob.Agents Chemother. **39**:2164-2166.

54. **Hinrichs, W., C. Kisker, M. Duvel, A. Muller, K. Tovar, W. Hillen, and W. Saenger**. 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. Science **264**:418-420.

55. **Honore, N. and S. T. Cole**. 1994. Streptomycin resistance in mycobacteria. Antimicrob.Agents Chemother. **38**:238-242.

56. **Huitric, E., J. Werngren, P. Jureen, and S. Hoffner**. 2006. Resistance levels and rpoB gene mutations among in vitro-selected rifampin-resistant Mycobacterium tuberculosis mutants. Antimicrob.Agents Chemother. **50**:2860-2862.

57. **Jadaun, G. P., R. Das, P. Upadhyay, D. S. Chauhan, V. D. Sharma, and V. M. Katoch**. 2009. Role of embCAB gene mutations in ethambutol resistance in Mycobacterium tuberculosis isolates from India. Int.J.Antimicrob.Agents **33**:483-486.

58. **Jarlier, V. and H. Nikaido**. 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol.Lett. **123**:11-18.

59. **Johnson, R., E. M. Streicher, G. E. Louw, R. M. Warren, P. D. van Helden, and T. C. Victor**. 2006. Drug resistance in Mycobacterium tuberculosis. Curr.Issues Mol.Biol. **8**:97-111.

60. **Jones, K. D., T. Hesketh, and J. Yudkin**. 2008. Extensively drug-resistant tuberculosis in sub-Saharan Africa: an emerging public-health concern. Trans.R.Soc.Trop.Med.Hyg. **102**:219-224.

61. **Kam, K. M., C. W. Yip, T. L. Cheung, H. S. Tang, O. C. Leung, and M. Y. Chan**. 2006. Stepwise Decrease in Moxifloxacin Susceptibility amongst Clinical Isolates of Multidrug-Resistant Mycobacterium tuberculosis: Correlation with Ofloxacin Susceptibility. Microb.Drug Resist. **12**:7-11.

62. **Larsen, M. H., C. Vilcheze, L. Kremer, G. S. Besra, L. Parsons, M. Salfinger, L. Heifets, M. H. Hazbon, D. Alland, J. C. Sacchettini, and W. R. Jacobs, Jr.** 2002. Overexpression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in Mycobacterium smegmatis, M. bovis BCG and M. tuberculosis. Mol.Microbiol. **46**:453-466.

63. **Lewis, M.** 2005. The lac repressor. C.R.Biol. **328**:521-548.

64. **Li, X. Z. and H. Nikaido**. 2004. Efflux-mediated drug resistance in bacteria. Drugs **64**:159-204.

65. **Li, X. Z., L. Zhang, and H. Nikaido**. 2004. Efflux pump-mediated intrinsic drug resistance in Mycobacterium smegmatis. Antimicrob.Agents Chemother. **48**:2415-2423.

66. **Liu, J., H. E. Takiff, and H. Nikaido**. 1996. Active efflux of fluoroquinolones in Mycobacterium smegmatis mediated by LfrA, a multidrug efflux pump. J.Bacteriol. **178**:3791-3795.

67. **Llano-Sotelo, B., E. F. Azucena, Jr., L. P. Kotra, S. Mobashery, and C. S. Chow**. 2002. Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. Chem.Biol. **9**:455-463.

68. **Madison, B. M., S. H. Siddiqi, L. Heifets, W. Gross, M. Higgins, N. Warren, A. Thompson, G. Morlock, and J. C. Ridderhof**. 2004. Identification of a Mycobacterium tuberculosis strain with stable, low-level resistance to isoniazid. J.Clin.Microbiol. **42**:1294-1295.

69. **Magalhaes, M. L., A. Argyrou, S. M. Cahill, and J. S. Blanchard**. 2008. Kinetic and mechanistic analysis of the Escherichia coli ribD-encoded bifunctional deaminase-reductase involved in riboflavin biosynthesis. Biochemistry **47**:6499-6507.

70. **Mariam, D. H., Y. Mengistu, S. E. Hoffner, and D. I. Andersson**. 2004. Effect of rpoB mutations conferring rifampin resistance on fitness of Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **48**:1289-1294.

71. **Maus, C. E., B. B. Plikaytis, and T. M. Shinnick**. 2005. Mutation of tlyA confers capreomycin resistance in Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **49**:571-577.

72. **Mdluli, K. and Z. Ma**. 2007. Mycobacterium tuberculosis DNA gyrase as a target for drug discovery. Infect.Disord.Drug Targets. **7**:159-168.

73. **Meier, A., P. Sander, K. J. Schaper, M. Scholz, and E. C. Bottger**. 1996. Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **40**:2452-2454.

74. **Mick, V., M. J. Rebollo, A. Lucia, M. J. Garcia, C. Martin, and J. A. Ainsa**. 2008. Transcriptional analysis of and resistance level conferred by the aminoglycoside acetyltransferase gene aac(2')-Id from Mycobacterium smegmatis. J.Antimicrob.Chemother. **61**:39-45.

75. **Miesel, L., T. R. Weisbrod, J. A. Marcinkeviciene, R. Bittman, and W. R. Jacobs, Jr.** 1998. NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in Mycobacterium smegmatis. J.Bacteriol. **180**:2459-2467.

76. **Molle, V., L. Kremer, C. Girard-Blanc, G. S. Besra, A. J. Cozzone, and J. F. Prost**. 2003. An FHA phosphoprotein recognition domain mediates protein EmbR phosphorylation by PknH, a Ser/Thr protein kinase from Mycobacterium tuberculosis. Biochemistry **42**:15300-15309.

77. **Morlock, G. P., B. Metchock, D. Sikes, J. T. Crawford, and R. C. Cooksey**. 2003. ethA, inhA, and katG loci of ethionamide-resistant clinical Mycobacterium tuberculosis isolates. Antimicrob.Agents Chemother. **47**:3799-3805.

78. **Morris, R. P., L. Nguyen, J. Gatfield, K. Visconti, K. Nguyen, D. Schnappinger, S. Ehrt, Y. Liu, L. Heifets, J. Pieters, G. Schoolnik, and C. J. Thompson**. 2005. Ancestral antibiotic resistance in Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A .

79. **Morris, S., G. H. Bai, P. Suffys, L. Portillo-Gomez, M. Fairchok, and D. Rouse**. 1995. Molecular mechanisms of multiple drug resistance in clinical isolates of Mycobacterium tuberculosis. J.Infect.Dis. **171**:954-960.

80. **Mulder, N. J., H. Zappe, and L. M. Steyn**. 1999. Characterization of a Mycobacterium tuberculosis homologue of the Streptomyces coelicolor whiB gene. Tuber.Lung Dis. **79**:299-308.

81. **Pagan-Ramos, E., J. Song, M. McFalone, M. H. Mudd, and V. Deretic**. 1998. Oxidative stress response and characterization of the oxyR-ahpC and furA-katG loci in Mycobacterium marinum. J.Bacteriol. **180**:4856-4864.

82. **Pasca, M. R., P. Guglierame, F. Arcesi, M. Bellinzoni, E. De Rossi, and G. Riccardi**. 2004. Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **48**:3175-3178.

83. **Pasca, M. R., P. Guglierame, E. De Rossi, F. Zara, and G. Riccardi**. 2005. mmpL7 gene of Mycobacterium tuberculosis is responsible for isoniazid efflux in Mycobacterium smegmatis. Antimicrob.Agents Chemother. **49**:4775-4777.

84. **Payton, M., R. Auty, R. Delgoda, M. Everett, and E. Sim**. 1999. Cloning and characterization of arylamine N-acetyltransferase genes from Mycobacterium smegmatis and Mycobacterium tuberculosis: increased expression results in isoniazid resistance. J.Bacteriol. **181**:1343-1347.

85. **Payton, M., C. Gifford, P. Schartau, C. Hagemeier, A. Mushtaq, S. Lucas, K. Pinter, and E. Sim**. 2001. Evidence towards the role of arylamine N-acetyltransferase in Mycobacterium smegmatis and development of a specific antiserum against the homologous enzyme of Mycobacterium tuberculosis. Microbiology **147**:3295-3302.

86. **Pym, A. S., P. Domenech, N. Honore, J. Song, V. Deretic, and S. T. Cole**. 2001. Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by furA of Mycobacterium tuberculosis. Mol.Microbiol. **40**:879-889.

87. **Quan, S., H. Venter, and E. R. Dabbs**. 1997. Ribosylative inactivation of rifampin by Mycobacterium smegmatis is a principal contributor to its low susceptibility to this antibiotic. Antimicrob.Agents Chemother. **41**:2456-2460.

88. **Ramaswamy, S. and J. M. Musser**. 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tuber.Lung Dis. **79**:3-29.

89. **Ramaswamy, S. V., A. G. Amin, S. Goksel, C. E. Stager, S. J. Dou, H. El Sahly, S. L. Moghazeh, B. N. Kreiswirth, and J. M. Musser**. 2000. Molecular genetic analysis of nucleotide

polymorphisms associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **44**:326-336.

90. **Ramaswamy, S. V., R. Reich, S. J. Dou, L. Jasperse, X. Pan, A. Wanger, T. Quitugua, and E. A. Graviss**. 2003. Single nucleotide polymorphisms in genes associated with isoniazid resistance in Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **47**:1241-1250.

91. **Ramon-Garcia, S., C. Martin, J. A. Ainsa, and R. E. De**. 2006. Characterization of tetracycline resistance mediated by the efflux pump Tap from Mycobacterium fortuitum. J.Antimicrob.Chemother. **57**:252-259.

92. **Ramon-Garcia, S., C. Martin, C. J. Thompson, and J. A. Ainsa**. 2009. The role of the Mycobacterium tuberculosis P55 efflux pump in intrinsic drug resistance, oxidative stress responses and growth. Antimicrob.Agents Chemother.

93. **Ramon-Garcia, S., I. Otal, C. Martin, R. Gomez-Lus, and J. A. Ainsa**. 2006. Novel streptomycin resistance gene from Mycobacterium fortuitum. Antimicrob.Agents Chemother. **50**:3920-3922.

94. **Rattan, A., A. Kalia, and N. Ahmad**. 1998. Multidrug-resistant Mycobacterium tuberculosis: molecular perspectives. Emerg.Infect.Dis. **4**:195-209.

95. **Riska, P. F., W. R. Jacobs, Jr., and D. Alland**. 2000. Molecular determinants of drug resistance in tuberculosis. Int.J.Tuberc.Lung Dis. **4**:S4-10.

96. **Roe, M. T. and S. D. Pillai**. 2003. Monitoring and identifying antibiotic resistance mechanisms in bacteria. Poult.Sci. **82**:622-626.

97. **Sala, C., F. Forti, E. Di Florio, F. Canneva, A. Milano, G. Riccardi, and D. Ghisotti**. 2003. Mycobacterium tuberculosis FurA autoregulates its own expression. J.Bacteriol. **185**:5357-5362.

98. **Sander, P., E. De Rossi, B. Boddinghaus, R. Cantoni, M. Branzoni, E. C. Bottger, H. Takiff, R. Rodriquez, G. Lopez, and G. Riccardi**. 2000. Contribution of the multidrug efflux pump LfrA to innate mycobacterial drug resistance. FEMS Microbiol.Lett. **193**:19-23.

99. **Savjani, J. K., A. K. Gajjar, and K. T. Savjani**. 2009. Mechanisms of resistance: useful tool to design antibacterial agents for drug - resistant bacteria. Mini.Rev.Med.Chem. **9**:194-205.

100. **Scorpio, A. and Y. Zhang**. 1996. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat.Med. **2**:662-667.

101. **Sharma, K., M. Gupta, M. Pathak, N. Gupta, A. Koul, S. Sarangi, R. Baweja, and Y. Singh**. 2006. Transcriptional control of the mycobacterial embCAB operon by PknH through a regulatory protein, EmbR, in vivo. J.Bacteriol. **188**:2936-2944.

102. **Shenoi, S. and G. Friedland**. 2009. Extensively drug-resistant tuberculosis: a new face to an old pathogen. Annu.Rev.Med. **60**:307-320.

103. **Sherman, D. R., P. J. Sabo, M. J. Hickey, T. M. Arain, G. G. Mahairas, Y. Yuan, C. E. Barry, III, and C. K. Stover**. 1995. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc.Natl.Acad.Sci.U.S.A **92**:6625-6629.

104. **Shi, R., N. Itagaki, and I. Sugawara**. 2007. Overview of anti-tuberculosis (TB) drugs and their resistance mechanisms. Mini.Rev.Med.Chem. **7**:1177-1185.

105. **Sholto-Douglas-Vernon, C., J. Sandy, T. C. Victor, E. Sim, and P. D. Helden**. 2005. Mutational and expression analysis of tbnat and its response to isoniazid. J.Med.Microbiol. **54**:1189-1197.

106. **Siddiqi, N., R. Das, N. Pathak, S. Banerjee, N. Ahmed, V. M. Katoch, and S. E. Hasnain**. 2004. Mycobacterium tuberculosis isolate with a distinct genomic identity overexpresses a tap-like efflux pump. Infection **32**:109-111.

107. **Sikora, A. L., B. A. Frankel, and J. S. Blanchard**. 2008. Kinetic and chemical mechanism of arylamine N-acetyltransferase from Mycobacterium tuberculosis. Biochemistry **47**:10781-10789.

108. **Silva, P. E., F. Bigi, S. M. de la Paz, M. I. Romano, C. Martin, A. Cataldi, and J. A. Ainsa**. 2001. Characterization of P55, a multidrug efflux pump in Mycobacterium bovis and Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **45**:800-804.

109. **Springer, B., Y. G. Kidan, T. Prammananan, K. Ellrott, E. C. Bottger, and P. Sander**. 2001. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. Antimicrob.Agents Chemother. **45**:2877-2884.

110. **Sreevatsan, S., X. Pan, K. E. Stockbauer, D. L. Williams, B. N. Kreiswirth, and J. M. Musser**. 1996. Characterization of rpsL and rrs mutations in streptomycin-resistant Mycobacterium tuberculosis isolates from diverse geographic localities. Antimicrob.Agents Chemother. **40**:1024-1026.

111. **Sreevatsan, S., K. E. Stockbauer, X. Pan, B. N. Kreiswirth, S. L. Moghazeh, W. R. Jacobs, Jr., A. Telenti, and J. M. Musser**. 1997. Ethambutol resistance in Mycobacterium tuberculosis: critical role of embB mutations. Antimicrob.Agents Chemother. **41**:1677-1681.

112. **Srivastava, K., R. Das, P. Jakhmola, P. Gupta, D. S. Chauhan, V. D. Sharma, H. B. Singh, A. S. Sachan, and V. M. Katoch**. 2004. Correlation of mutations detected by INNO-LiPA with levels of rifampicin resistance in Mycobacterium tuberculosis. Indian J.Med.Res. **120**:100-105.

113. **Srivastava, S., A. Garg, A. Ayyagari, K. K. Nyati, T. N. Dhole, and S. K. Dwivedi**. 2006. Nucleotide polymorphism associated with ethambutol resistance in clinical isolates of Mycobacterium tuberculosis. Curr.Microbiol. **53**:401-405.

114. **Storz, G. and S. Altuvia**. 1994. OxyR regulon. Methods Enzymol. **234**:217-223.

115. **Storz, G., L. A. Tartaglia, and B. N. Ames**. 1990. The OxyR regulon. Antonie Van Leeuwenhoek **58**:157-161.

116. **Takayama, K. and J. O. Kilburn**. 1989. Inhibition of synthesis of arabinogalactan by ethambutol in Mycobacterium smegmatis. Antimicrob.Agents Chemother. **33**:1493-1499.

117. **Takiff, H. E., M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. Jacobs, Jr.** 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in Mycobacterium smegmatis. Proc.Natl.Acad.Sci.U.S.A **93**:362-366.

118. **Tanaka, Y., K. Yazawa, E. R. Dabbs, K. Nishikawa, H. Komaki, Y. Mikami, M. Miyaji, N. Morisaki, and S. Iwasaki**. 1996. Different rifampicin inactivation mechanisms in Nocardia and related taxa. Microbiol.Immunol. **40**:1-4.

119. **Taniguchi, H., H. Aramaki, Y. Nikaido, Y. Mizuguchi, M. Nakamura, T. Koga, and S. Yoshida**. 1996. Rifampicin resistance and mutation of the rpoB gene in Mycobacterium tuberculosis. FEMS Microbiol.Lett. **144**:103-108.

120. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer**. 1993. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet **341**:647-650.

121. **Telenti, A., W. J. Philipp, S. Sreevatsan, C. Bernasconi, K. E. Stockbauer, B. Wieles, J. M. Musser, and W. R. Jacobs, Jr.** 1997. The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. Nat.Med. **3**:567-570.

122. **Upton, A. M., A. Mushtaq, T. C. Victor, S. L. Sampson, J. Sandy, D. M. Smith, P. V. van Helden, and E. Sim**. 2001. Arylamine N-acetyltransferase of Mycobacterium tuberculosis is a polymorphic enzyme and a site of isoniazid metabolism. Mol.Microbiol. **42**:309-317.

123. **van Soolingen, D., P. E. de Haas, H. R. van Doorn, E. Kuijper, H. Rinder, and M. W. Borgdorff**. 2000. Mutations at amino acid position 315 of the katG gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of Mycobacterium tuberculosis in the Netherlands. J.Infect.Dis. **182**:1788-1790.

124. **Vannelli, T. A., A. Dykman, and P. R. Ortiz de Montellano**. 2002. The antituberculosis drug ethionamide is activated by a flavoprotein monooxygenase. J.Biol.Chem. **277**:12824-12829.

125. **Velayati, A. A., M. R. Masjedi, P. Farnia, P. Tabarsi, J. Ghanavi, A. H. Ziazarifi, and S. E. Hoffner**. 2009. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran. Chest **136**:420-425.

126. **Viveiros, M., C. Leandro, and L. Amaral**. 2003. Mycobacterial efflux pumps and chemotherapeutic implications. Int.J.Antimicrob.Agents **22**:274-278.

127. **Whittle, G., N. B. Shoemaker, and A. A. Salyers**. 2002. The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. Cell Mol.Life Sci. **59**:2044-2054.

128. **Wilson, C. J., H. Zhan, L. Swint-Kruse, and K. S. Matthews**. 2007. The lactose repressor system: paradigms for regulation, allosteric behavior and protein folding. Cell Mol.Life Sci. **64**:3-16.

129. **Wilson, M., J. DeRisi, H. H. Kristensen, P. Imboden, S. Rane, P. O. Brown, and G. K. Schoolnik**. 1999. Exploring drug-induced alterations in gene expression in Mycobacterium tuberculosis by microarray hybridization. Proc.Natl.Acad.Sci.U.S.A **96**:12833-12838.

130. **Wright, G. D. and P. R. Thompson**. 1999. Aminoglycoside phosphotransferases: proteins, structure, and mechanism. Front Biosci. **4**:D9-21.

131. **Yew, W. W. and C. C. Leung**. 2008. Management of multidrug-resistant tuberculosis: Update 2007. Respirology. **13**:21-46.

132. **Zaczek, A., A. Brzostek, E. ugustynowicz-Kopec, Z. Zwolska, and J. Dziadek**. 2009. Genetic evaluation of relationship between mutations in rpoB and resistance of Mycobacterium tuberculosis to rifampin. BMC.Microbiol. **9**:10.

133. **Zahrt, T. C., J. Song, J. Siple, and V. Deretic**. 2001. Mycobacterial FurA is a negative regulator of catalase-peroxidase gene katG. Mol.Microbiol. **39**:1174-1185.

134. **Zainuddin, Z. F. and J. W. Dale**. 1990. Does Mycobacterium tuberculosis have plasmids? Tubercle. **71**:43-49.

135. **Zeng, L. and S. Jin**. 2003. aph(3')-IIb, a gene encoding an aminoglycoside-modifying enzyme, is under the positive control of surrogate regulator HpaA. Antimicrob.Agents Chemother. **47**:3867-3876.

136. **Zhang, N., J. B. Torrelles, M. R. McNeil, V. E. Escuyer, K. H. Khoo, P. J. Brennan, and D. Chatterjee**. 2003. The Emb proteins of mycobacteria direct arabinosylation of lipoarabinomannan and arabinogalactan via an N-terminal recognition region and a C-terminal synthetic region. Mol.Microbiol. **50**:69-76.

137. **Zhang, Y.** 2005. The magic bullets and tuberculosis drug targets. Annu.Rev.Pharmacol.Toxicol. **45**:529-564.

138. **Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole**. 1992. The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature **358**:591-593.

CHAPTER 3

MATERIALS AND METHODS

A three pronged approach, using proteomics, transcriptomic and genomic methods were used to enhance the possibility to identify mechanisms which can explain varying levels of RIF resistance in two closely related clinical isolates. An outline of the experimental procedures is given in Figure 3.1.

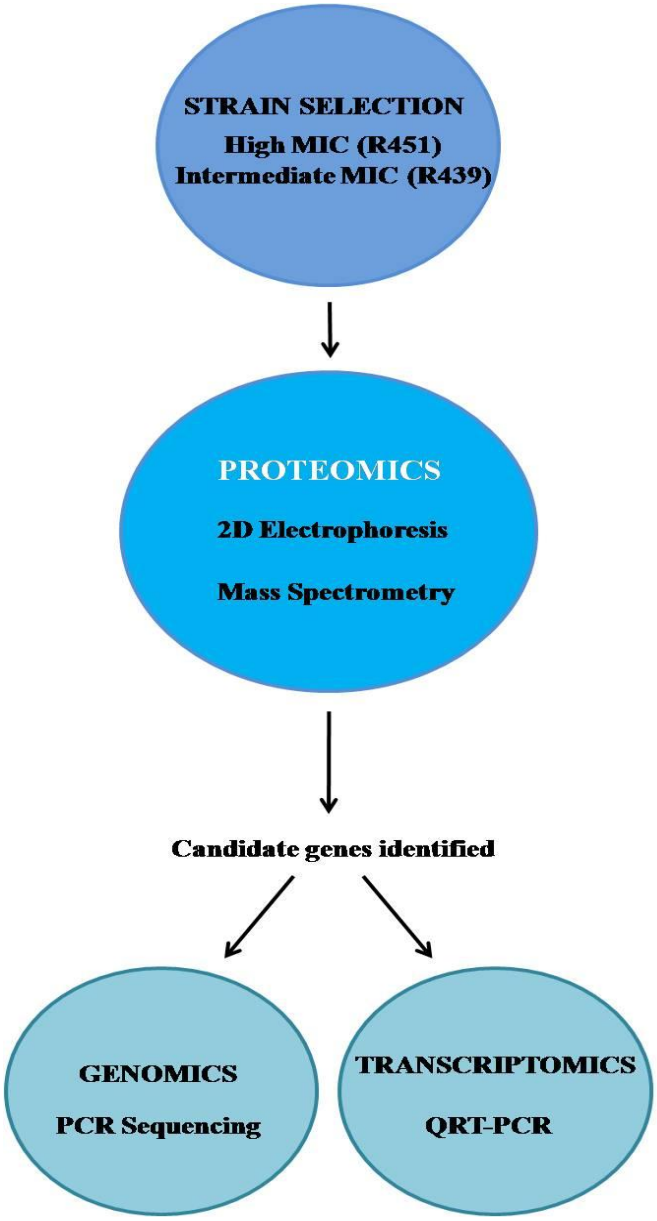

Figure 3.1: Summary of techniques used in this study

3.1. STRAIN SELECTION

Well characterized clinical isolates (R439 and R451) of *M. tuberculosis* was selected from an existing local sample bank maintained at the Stellenbosch University, Western Cape, South Africa. In this setting three dominant strain groups are present: Beijing, Low Copy Clade (LCC) and LAM (Latin-American and Mediterranean) (5). These two clinical isolates are from the same LCC evolutionary lineage (identical *IS6110* RFLP and spoligotype fingerprints) and were selected based on the having identical mutations in the *katG* (315 ACA), *emb* (306 ATA), *pncA* (Del58) and *rpoB* gene (531 TTG) with intermediate (R439) and high (R451) levels of RIF resistance (Table 3.1) (PhD thesis, Gail E. Louw, 2009). No low MIC strain was selected as isolates were exposed to 2ug/ml, which would have a killing effect on the low MIC strain. MICs are defined as the lowest concentration of the compound capable of inhibiting visible growth of a microorganism after overnight incubation (1,3).

Table 3.1: Characteristics of selected clinical isolates

Isolate	Family	Spoligotype	Cluster	IS6110 RFLP	RIF MIC (ug/ml)
R439[a]	LCC	115	DRF150		60
R451[b]					170

Legend to table 4.3: a - LCC RIF resistant isolate with intermediate RIF resistance levels;
b - LCC RIF resistant isolate with high RIF resistance levels

3.2. CULTIVATION OF *M. TUBERCULOSIS* STRAINS

Preparation of freezer stock:

Strains were first grown on Lowenstein-Jensen (LJ) solid medium with continuous aeration for approximately three weeks at 37°C. A starter culture was set up by scraping "buff" colored colonies from LJ's slants and inoculating it into 5 ml of 7H9 Middlebrook medium (Becton, Dickinson Microbiology system, Sparks, USA), supplemented with 10% albumin-dextrose-catalase (ADC), with 0.2% (v/v) glycerol (Merck Laboratories, Saarchem, Gauteng, SA) and 0.1% Tween80 (Becton, Microbiology systems, Sparks, USA). Cultures were then grown in filtered screw cap tissue culture flasks (Greiner Bio-one, Maybachstreet, Germany) without shaking for 37°C until an optical density (OD_{600}) of 0.6-0.8 were

reached. The cultures were then inspected for contamination by Ziehl-Neelsen (ZN) staining and cultured onto blood agar plates. The primary cultures were subsequently sub-cultured in 10 ml 7H9 Middlebrook medium supplemented with ADC and incubated at 37°C. After an OD of 0.7-0.8 has been reached the cultures were re-inspected for contamination before 50% glycerol stocks (1:1 v/v, 500µl culture and 500µl 50% glycerol) was prepared and stored at -80°C.

ZN staining and Blood agar plates:

The presence of contaminants in the *M. tuberculosis* cultures were continuously monitored with ZN staining as well as plating the culture onto a blood agar for two days (*M. tuberculosis* does not grow on blood agar within 2 days). For ZN staining a *M. tuberculosis* smear was heat fixed (heated for 2 hours at 100 °C), stained with carbol-fuchsin (Becton, Dickinson and Company, Maryland, USA), and decolorized with acid alcohol. The smear was then counterstained with methylene blue (Becton, Dickson and Company, Maryland, USA) and read under the microscope for acid-fast bacilli. *M. tuberculosis*, an acid-fast bacterium, will retain dyes when heated and treated with acidified organic compounds. Therefore it will appear pink in a contrasting background when the ZN test is done.

Setting up initial cultures for Protein and RNA experiments

Cultures for both protein (1600ul stock into 160ml media) and RNA (60ul stock into 60ml media) extractions was set up by making a 1:100 dilution of the freezer stock into 7H9 Middlebrook media supplemented with ADC. Cultures were incubated in tissue culture flasks at 37°C until an OD_{600} of 0.7-0.8 has been reached. Liquid cultures were then divided into two portions: one half of the culture served as control, where as the remaining half of the culture was exposed to the critical concentration of Rifampicin (RIF) (Becton, Dickinson and Company, Sparks, USA) (2 µg/ml) and incubated at 37 ºC for a further 24 hours after which either protein or RNA was extracted. The critical concentration is defined as the drug concentration used to differentiate between drug susceptible and drug resistant isolates. An isolate is determined as resistant when 1% or more of the test population grows in the presence of the critical concentration of the drug. This concentration was used as it would not result in a killing effect of high and intermediate level RIF resistant isolates. Two biological (to assess biological measurements for two independent experiments done on different days) and 2 technical replicates (repeated measures of one biological sample on the same day) was done for all isolates.

3.3. PROTEOMICS

3.3.1. Membrane protein extractions of *M. tuberculosis*

Cultures were centrifuged (Eppendorf, Centrifuge 5810R) in 50ml tubes (LASEC, South Africa) at 3000 rpm for 20 minutes at 20ºC. The supernatant was discarded and the pellet re-suspended in 1 ml Phosphate buffer saline (PBS), pH 7.4, containing 1% (v/v) Tween80 (Merck Laboratories, Saarchem, Gauteng, SA) in a 2 ml screw cap tube (Whitehead Scientific, South Africa). The suspension was then centrifuged for a further 5 minutes at 6000 rpm. The supernatant was discarded and the resulting pellet was re suspended in 1 ml PBS. The suspension was centrifuged for a further 5 minutes at 6000 rpm. After centrifugation the supernatant was discarded and an equal volume of glass beads (0.1 mm, Separations, South Africa) and approximately 200-300 µl lysis buffer was added to the bacterial pellet. The bacterial cells were ribolysed using a FastPrep FP120 ribolyser (Bio101 SAVANT, Vista, USA) at 6.5m/s for 2 x 45 seconds with 1 minute of cooling on ice between intervals. The lysed suspension were then incubated on ice for 10 minutes and centrifuged for 15 minutes at 13 000 rpm. The supernatant, containing the whole-cell lysate proteins were then filter-sterilized with a 0.22 µm Millex-GV filter (Millipore, Bangalore, India) and transferred to a clean 1.5ml Eppendorf tube (Merck, New Jersey, USA).

The recovered whole-cell lysate (WCL) proteins were fractionated into cell wall, cytosol and membrane proteins. The whole-cell lysate proteins were centrifuged for 1 hour at 17 000 rpm. After centrifugation the supernatant were transferred to a clean 1.5ml Eppendorf tube (Merck, New Jersey, USA) and the pellet, containing cell wall proteins, was re-suspended in 150 µl lysis buffer. The supernatant were then centrifuged for 2 hours at 33 000 rpm. After centrifugation the supernatant, containing the cytosol proteins, were transferred to a new tube and the pellet, containing the membrane proteins were re suspended in 150 µl lysis buffer. The different protein fractions were stored at -20°C until needed for further analysis. Previous work (PhD thesis, Gail E. Louw, 2009) has shown that genes encoding proteins, specifically ABC transporters, are significantly differentially expressed after RIF exposure. Therefore only membrane proteins were used in subsequent experiments.

3.3.2. Determination of protein concentrations and purification

Protein concentrations were determined with the RC DC Protein assay (Bio-Rad Laboratories, Hercules, CA 94547) according to the manufacturer's instructions. The proteins were purified using the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories, Hercules, CA 94547) according to the manufacturer's instructions.

3.3.3. Protein separation

Isoelectric focusing (IEF) – First dimension separation

Two hundred µg of the purified membrane protein sample was suspended in ReadyPrep 2-D Rehydration buffer 1(Bio-Rad Laboratories, Hercules, CA 94547) containing Bio-lite pH 3-10 buffer (Bio-Rad Laboratories, Hercules, CA 94547), to a final volume of 200ul. The protein/rehydration buffer mix was then applied to a pH 4-7 (11 cm) immobilised pH gradient (IPG) strip (Bio-Rad Laboratories, Hercules, CA 94547) and allowed to rehydrate at room temperature for at least 12 hours. IEF was done under the following conditions: 8000 V for 20 min, 8000 V for 2 hours and 8000 V for 40 000 V hours at 21°C (Protean IEF cell, Bio-Rad Laboratories, Hercules, CA 94547). After IEF focusing the strips were stored at -80^{o}C until required.

2-Dimensional gel electrophoresis (2-DE)

Equilibration of IPG strips was done by incubating the strips sequentially in equilibration buffer containing DTT (Sigma-Aldrich, Missouri, USA) for 15 minutes and then in equilibration buffer containing Idoacetamide (Sigma –Aldrich, Missouri, USA) for 15 minutes at room temperature. After equilibration, the IPG strips were placed on top of the 12% 2-D SDS-PAGE gels, and sealed with 0.5% agarose (Bio-Rad Laboratories, Hercules, CA 94547). Gel electrophoresis of the second dimension separation was done under constant voltage (250 V) for 2-3 hours in cathode and anode buffers.

3.3.4. Protein detection using coomassie brilliant blue

After 2-DE, the proteins were fixed in the gel by incubating in a fixing solution for 1 hour. Protein gels were then heated for 2 min in Coomassie solution 1 before overnight incubation. After overnight incubation, gels were immersed and heated in Coomassie solution 2 for 2 minutes after which it was placed on a shaker for 30 minutes. This was repeated with Coomassie solution 3. The gels were then washed in a destaining solution until complete destaining was achieved. The gels were stored in a 25% ammonium sulfate solution (Merck, Darmstadt, Germany) at room temperature until further use.

3.3.5. Protein spot identification and comparisons on 2-DE gels

Coomassie stained gels were digitalized using a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA 94547) and image analysis were done with Quantityone and PDQuest 2-D advanced 2-D analysis software (Version 8.0) (Bio-Rad Laboratories, Hercules, CA 94547). All 2-DE experiments were repeated at least three times from independent initial cultures to overcome technical and biological variation.

Three independent comparative analyses were done on gels generated by 2-DE from the two selected strains: (i) High MIC strain (R451) RIF exposed VS High MIC strain (R451) unexposed, (ii) Intermediate MIC strain (R439) RIF exposed VS Intermediate (R439) MIC unexposed strain, (iii) High MIC strain (R451) RIF exposed VS Intermediate MIC strain (R439) RIF exposed. No comparison between the unexposed strains was made, as the aim of this study was to observe the proteomic response of these isolates when exposed to RIF.

A Master Image Gel (MIG) was constructed from all triplicate gels and utilized for comparison. All spots constantly present on all three gels were included on the master gel and used for quantitative and qualitative analysis. Statistical spot-intensity comparison between the two strains between different experimental conditions was conducted with a PDQuest integrated T-test with a confidence interval of 95%. A significant level of $p<0.05$ was considered as statistically significant. Quantitative (present and absent) and qualitative (differentially expressed) analysis was also done visually to refine stringency. Spots of interest (spots statistically differentially expressed between the two strains between different experimental conditions) were excised with sterile blades from gels and stored in 100µl of double distilled

Milli-Q water in a 1.5ml Eppendorf tube (Merck, New Jersey, USA) at room temperature for future Mass spectrometric analysis.

3.3.6. Protein identification

Mass Spectrometry (MS) analysis was done by the Proteomic Research Group at the University of the Western Cape. Briefly, excised gel spots were digested with trypsin prior to MALDI-TOF analysis. The peptide masses generated with Matrix Assisted Lazer Desorption/Ionization Time of Flight (MALDI-TOF) analysis were then submitted into the Mascot program (http://www.matrixscience.com). Protein MOWSE scores produced by the mascot program greater than 52 were considered as significant. Results obtained from MALDI-TOF mass spectrometry were further compared to the whole genome sequence comparison of the *M. tuberculosis* H37Rv reference strain (http://genolist.pasteur.fr/TubercuList) and categorized according to function from literature searches (1).

3.4. TRANSCRIPTOMICS

3.4.1. RNA Extractions from *M. tuberculosis*

After antibiotic exposure, five volumes of 5M GITC (Sigma-Aldrich, St Louis, Germany) were added to one volume of each *M. tuberculosis* culture. The cultures were centrifuged (Eppendorf, Centrifuge 5810R) in 50 ml tubes (LASEC, South Africa) at 3 000 rpm for 20 minutes at 20 °C. The supernatant was discarded and the bacterial pellet was transferred to a 2 ml Eppindorf tube (Merck, New Jersey, USA) and centrifuged at 13 000 rpm for 1 minute at room temperature. After centrifugation, the pellet was re-suspended in 1 ml TRIZOL LS reagent (Invitrogen, CA, USA). The suspension was transferred to a 2 ml screw capped tube containing silica beads (IEPSA, Medical diagnostics, South Africa) and ribolysed using a FastPrep FP120 ribolyser (Bio101 SAVANT, Vista, USA) at 6 m/s for 3 x 20 seconds with 1 minute of cooling on ice between intervals. The lysed suspension was centrifuged at 13 000 rpm for 45 seconds. The supernatant above the beads and cellular debris were transferred to an 2 ml Phase Lock gel tube (Merck New Jersey, USA) containing 300µl chloroform: Isoamyl alcohol (24:1) (Sigma-Aldrich, St Louis, Germany). The tube was inverted rapidly for 15 seconds, followed with periodically inversion for an additional 2 minutes. The tube was incubated for 5 minutes at room temperature and then centrifuged at 13 000 rpm for 10 minutes. The top aqueous layer was then transferred to a clean 1.5 ml tube containing an equal volume of isopropanol and incubated overnight at -20°C. Precipitated nucleic acids were collected by centrifugation at 12 000g for 30 minutes at 4ºC. After centrifugation, the RNA pellet was washed with 1 ml 70% ethanol, inverted several times and centrifuged at 12 000 g for 10 minutes at 4°C. After centrifugation, the ethanol was aspirated and the RNA pellet was allowed to air-dry at room temperature. The RNA pellet was dissolved in 70 µl RNase-free water (Ambion, Applied Biosystems, CA, USA) and stored at -80°C for further use.

DNase treatment

Contaminating chromosomal DNA was digested with RQ1 RNase-free DNase (Promega, WI, USA) according the instructions of the manufacturers. In short, 15 µl of RNA was added to 4 µl DNase and 4 µl DNase Buffer, followed by incubation for 30 minutes at 37°C. After incubation the DNase treated RNA was stored at -80°C for further use.

Phenol treatment

Traces of DNA were further eliminated by adding RNase-free water to the DNase treated sample to a final volume of 200 µl. An equal volume of Phenol: Chloroform (4:1) (Sigma-Aldrich, St Louis, Germany) was added to the diluted RNA and mixed by gentle inversion. The RNA/Phenol: Chloroform mix was incubated for 10 minutes on ice. After incubation, the RNA/Phenol: Chloroform mix was centrifuged for 10 minutes at 12 000 rpm at room temperature. The top layer was transferred to a clean tube and 1/10 volume of Sodium Acetate (pH 5.2) (Merck, New Jersey, USA) and 2.5 volumes of 100% Ethanol (Merck, New Jersey, USA) was added to the RNA. The RNA was incubated for 1 hour (or overnight) at -20°C. The precipitated nucleic acids were collected by centrifugation (12000 g for 30 minutes at 4°C). After centrifugation, the pellet was washed with 1 ml 70% ethanol, inverted several times and centrifuged at 12 000 g for 10 minutes at 4°C. After centrifugation, the ethanol was aspirated and the purified RNA pellet was allowed to air-dry at room temperature. The RNA pellet was dissolved in 70 µl of RNase-free water and stored at -80°C till further use. The RNA quality and quantity were assessed by using the Experion analyzer with the Experion Software version 2.01 (Bio-Rad Laboratories, Hercules, CA 94547).

3.4.2. cDNA Synthesis

cDNA was synthesized from 1µg highly purified RNA using the QuantiTect Reverse Transcriptase kit according to the manufacturer's instructions (Southern Cross Biotechnologies). In short, 1µg RNA was mixed with 2 µl genomic DNA Whipeout Buffer and RNase-free water (to a volume of 14 µl) and incubated for 2 minutes at 42°C. After incubation Quantiscript Reverse Transcriptase, Quantiscript RT buffer, and RT Primer Mix was added to the RNA mix and incubated for 15 minutes at 42°C. After incubation the mix was incubated for 3 minutes at 95ºC to inactivate the Quantiscript Reverse Transcriptase.

3.4.3. Primer design for Quantitative Real Time PCR (QRT-PCR) of candidate genes

PCR primers (Table 3.2) for quantitative reverse transcriptase analysis of the candidate genes were designed against the whole genome sequence of the *M. tuberculosis* H37Rv reference strain (http://genolist.pasteur.fr/TubercuList) using Primer software 3 version 0.2 (Whitehead Scientific, South Africa).

Table 3.2: Primers used for QRT-PCR

Gene	Primer name	Primer sequence	Product Size (bp)	Tm (°C)
atpA	atpA RT	Forward: 5' ATCCAGAGCGCAATCGAAG 3' Reverse: 5' GGGAATTCGAGCAGCTCTT 3'	150	61
atpH	atpH RT	Forward: 5' CGCATCGCAGAACAACTAGA 3' Reverse: 5' CTGATCGGCCACGTGATT 3'	182	60

3.4.4. Quantitative REAL-TIME PCR (QRT-PCR)

Currently two major real time PCR quantification methods are being used, the absolute or relative quantitative method (2,4) . Analysis of both methods can either be done using the standard curve method or the delta-delta Ct method. The standard curve method for either absolute or relative quantification is based on the construction of standard curve of cycle number at a threshold (C_T) against initial input amount of total RNA or copy number. This method assumes an approximately equal amplification efficiency of PCR among the diluted samples of the same gene, thus producing a linear relationship of C_T against initial input amount of total RNA or copy number. An optimal PCR reaction yields a slope of -3.3 on the standard curve with an ideal correlation coefficient value -1. Relative quantification using the delta-delta C_T method is the analytic method of choice for many real-time PCR studies. In this method a comparison within a sample (cDNA) is made with the gene of interest to that of a control gene. Quantification is done relative to the control gene(s) by subtracting the cycle threshold, or C_T, of the control gene from the C_T of the gene of interest ($R=2^{-(\Delta CT\ sample\ -\Delta CT\ control)}$), and the resulting difference in cycle number representing the fold difference of template for these two genes (4).

In this study both the standard curve and delta-delta C_T methods were used. The standard curve method was used for relative quantification of the reference genes (*SigA* and *16S)*. The template concentrations were given arbitrary values of 1 (for 10 fold-dilution of synthesized cDNA), 0.1 (for 100 fold dilution), 0.01(for 1 000 fold dilution) and 0.0001 (for 10 000 fold dilution). A standard curve assay was done for each reference gene on both biological and technical duplicate samples. Whereas the delta-delta C_T method was used for the candidate genes.

A standard 20µl PCR reaction was set up by adding 2µl of LightCycler Faststart DNA Master^{PLUS} SYBR Green I reaction mix (Roche Applied Science, Germany), 1 µl forward and reverse primer (10µM) of each candidate gene, 14µl of RNase-free water and 2µl of diluted cDNA (1/10 dilution for RIF treated samples, ½ dilution for control samples). A negative control was included which contained the same mix with the cDNA replaced by water. The PCR mix was subsequently transferred to capillary tubes (Roche Applied Science) in a pre-cooled adapter block after which it was transferred to a bench top centrifuge and centrifuged at 3rpm for 2 sec. The capillaries were placed into the Lightcycler 2.0 instrument (Roche Applied Science) and cycled through a four step PCR parameter protocol: (i) Activation program (95°C for 15 minutes); (ii) RT PCR program repeated for 50 cycles (95°C for 3s for denaturation, 60°C for 15s for annealing and 72°C for 6s for polymerase elongation); (ii) Melting curve program (95°C for 0s, 60°C for 15s, 90°C for 0s with a heating rate of 0.2°C/s) and (iv) Cooling down program of 40°C for 30s.

Therefore, for each gene a PCR reaction was set up with a 1000 fold dilution of cDNA of each reference gene (in duplicate), a two fold dilution of cDNA for control samples (in duplicate) and a 10 fold dilution of cDNA for RIF treated samples. Each PCR reaction was set up in for biological and technical duplicate samples.

3.4.5. Statistics

Significant fold changes were identified based on The Relative Expression Software Tool -384 (REST-384©) that assigns significance with a significance level of 5% (3).

3.5. GENOMICS

A panel of susceptible and resistant isolates from the Beijing ($n_{resistant}$=5, $n_{susecptible}$=5), LAM ($n_{resistant}$=5, $n_{susecptible}$=5), and LCC ($n_{resistant}$=5, $n_{susecptible}$=10) strain families were selected to screen for mutations that might be present in the candidate genes and in the regions immediate upstream of these genes.

3.5.1. DNA extraction

Crude DNA was extracted by boiling 500µl aliquot of the glycerol *M. tuberculosis* stock of each strain in a 1.5 ml Eppendorf tube (Merck, New Jersey, USA) for 20 minutes at 100°. These crude DNA templates were then stored for subsequent PCR analysis.

3.5.2. Primer design for PCR amplication of candidate genes

Primer software 3 version 0.2 (Whitehead Scientific, South Africa) and the *M. tuberculosis* H37Rv reference strain (http://genolist.pasteur.fr/TubercuList) were used to design four sets of overlapping PCR primers to amplify the complete DNA sequence of each candidate gene, as well as at least 90 base pairs upstream from the transcription start site (Figure 3.2; Table 3.3).

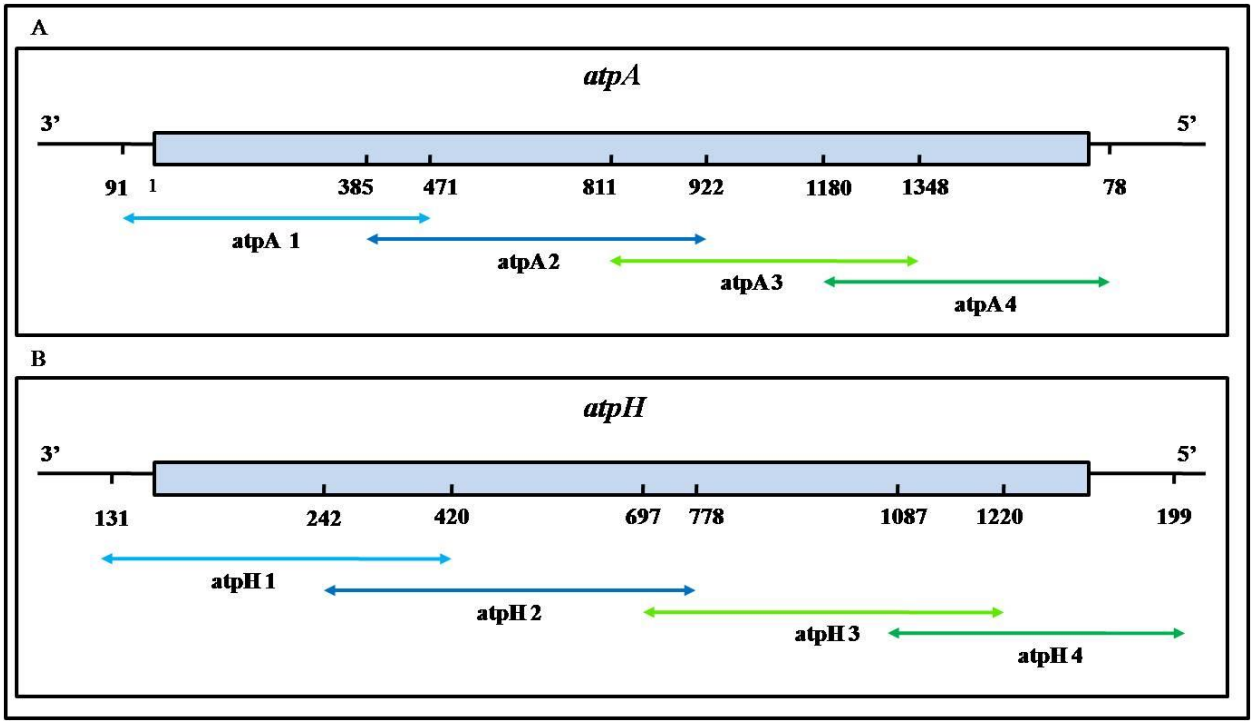

Figure 3.2: Primer design for candidate genes. For each candidate gene four sets of primers were designed for sequence analysis.

Table 3.3: Primers used for genomic PCR reactions

Gene	Primer name	Product region	Primer sequence	Product Size (bp)	Tm (°C)
atpA	atpA 1	- 91 to 471[a]	Forward: 5' ACGCTCTCGTCTCGTCTAGC 3' Reverse: 5' CGTCAATCGCCTTGATCC 3'	562	61
	atpA 2	385 to 922	Forward: 5' GAGACGTCGACTCCGATACTC 3' Reverse: 5' CGCGAATGCAGATAGAACAC 3'	537	61
	atpA 3	811 to 1348	Forward: 5' TTCGACGACCTGACTAAGCA 3' Reverse: 5' GGAAGATCGAAACCACTTGC 3'	537	61
	atpA 4	1180 to +78[b]	Forward: 5' GCTTGGACCTTTCGCAATAC 3' Reverse: 5' GGCCTTGGTGATCTTTTTGA 3'	548	61
atpH	atpH 1	- 131 to 420[c]	Forward: 5' AGCAATTGAAGCGGGAGAG 3' Reverse: 5' CTGATCGGCCACGTGATT 3'	551	60
	atpH 2	242 to 778	Forward: 5 'CTGATCGGCCACGTGATT 3' Reverse: 5' GCTTCGATACGGCTGTGC 3'	536	60
	atpH 3	697 to 1220	Forward: 5' GCTTCGATACGGCTGTGC 3' Reverse: 5' GTCACGGGGTGACCGTAG 3'	523	60
	atpH 4	1087 to +199[d]	Forward: 5' CTGTTCCTGGCCGAAGTTG 3' Reverse: 5' GTCATCACCGATGGCAAAC 3'	453	60

Legend to Table 3.3:
[a] Product region between 91 nucleotides upstream from transcription start site and 471 nucleotides inside gene
[b] Product region between 1180 nucleotides inside the structural gene to 78 nucleotides downstream from coding region
[c] Product region between 131 nucleotides upstream from transcription start site and 420 nucleotides inside gene
[d] Product region between 1087 nucleotides inside the structural gene to 199 nucleotides downstream from coding region

3.5.3. PCR Amplification of candidate genes

DNA amplifications were executed in 25 µl reaction volumes. The PCR master-mix consisted of 5 µl 5X Q-solution, 2.5 µl 10X reaction buffer, 2 µl $MgCl_2$ (25mM), 4 µl deoxyribonucleotide triphosphates (dNTPs) (0.2mM of each dNTP), 0.5 µl Forward primer (50pM), 0.5 µl Reverse primer (50pM), 0.125 µl Hotstar Taq polymerase (5 units/µ)l, 1.25 µl of the crude DNA template, and 7.85 dH_2O to make up a final volume of 25 µl. The reaction mixtures were heated in the thermal cycler (GeneAmp PCR System 2400, Applied Biosystems, Foster City, CA, USA) at the following conditions: an initial denaturing step at 95ºC for 15 minutes, followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at T_m (62ºC) for 1 minute, extension at 72ºC for 2 minutes, and a final extension step at 72ºC for 15 minutes. The primers used for the PCR reactions are tabulated in Table 3.3.

A 1.5% Agarose gel was prepared by dissolving 1.5 g Agarose in 100 ml 1x-TBE buffer with 5 µl ethidiumbromide. The PCR products were mixed with 5 µl loading dye (0.25% Xylene Cyanol, 30% glycerol) and loaded onto the gel. The gel was run at 150 V for approximately 1-2 hours in 1x TBE buffer and visualized under ultra violet light using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Vilber Lourmat, France).

3.5.4. Sequencing

PCR products were submitted for PCR clean-up and sequencing at the Central Analytic Facility of Stellenbosch University. Sequences were then compared with the whole genome sequence of *M. tuberculosis* H37Rv reference (http://genolist.pasteur.fr/TubercuList) to determine if mutations are present in the candidate genes from different *M. tuberculosis* strain families.

3.6. LIST OF BUFFERS AND SOLUTIONS

PCR BUFFERS

10X TE Buffer:
10mM Tris
1mM EDTA
dH_2O

10X TBE Buffer (pH 8.3):
0.45M Tris (108g)
0.44M Boric acid (55g)
10mM EDTA (7.4g)
dH_2O to make up 1L

CULTIVATION OF *M. TUBERCULOSIS* STRAINS

Middlebrook 7H9 medium:
7H9 medium (4.7g)
dH_2O (900ml)
Glycerol (2ml)
Polysorbate (Tween80) (0.5ml)

ADC:
BSA (25g)
Glucose (10g)
Catalase (0.75ml)
dH_2O to make up 500ml

EXTRACTION OF WCL PROTEINS

Lysis buffer:
0.5M Tris-HCl (Sigma-Aldrich, Missouri, USA) (5ml)
Triton X-114 (1ml)
10mM PMSF (1.74mg PMSF/1ml isopropanol) (5ml)
2 Protease inhibitor cocktail tablets (Roche Applied Science , Germany)
dH_2O to make up 50 ml

PBS (pH 7.3):

NaCl (8g)
KCI (0.2g)
diNaHPO$_4$ (1.15g)
KH$_2$PO$_4$ (0.2g)
1% Tween80
dH$_2$O to make 1L

2-D GEL ELECTROPHORESIS SOLUTIONS

Equilibration buffer 1:

0.375M Tris-HCl (pH 8.8)
6M Urea
30% Glycerol (Sigma-Aldrich, Missouri, USA)
2% SDS
2% DTT

Equilibration buffer 2:

0.375M Tris-HCl (pH 8.8) (Sigma-Aldrich, Missouri, USA)
6M Urea
30% Glycerol (Sigma-Aldrich, Missouri, USA)
2% SDS
1 g Iodoacetamide

Cathode buffer:

192M Glycine (pH 8.3) (Sigma-Aldrich, Missouri, USA)
pH with Tris (Sigma-Aldrich, Missouri, USA)
add 0.1% SDS
miliQH$_2$O to make 1L

Anode buffer:

0.375M Tris (pH 8.8) (Sigma-Aldrich, Missouri, USA)
pH with AcOH (Merck, Darmstadt, Germany)
miliQH$_2$O to make up 1.5L

Fixing buffer:	50% MeOH (Merck, Darmstadt, Germany) 5% AcOH (Merck, Darmstadt, Germany)
Coomassie I:	0.025% (w/v) Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Missouri, USA) 25% (v/v) isopropanol (Merck, Darmstadt, Germany) 10% (v/v) acetic acid (Merck, Darmstadt, Germany)
Coomassie II:	0.003% (w/v) Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Missouri, USA) 10% (v/v) isopropanol (Merck, Darmstadt, Germany) 10% (v/v) acetic acid (Merck, Darmstadt, Germany)
Coomassie III:	0.003% (w/v) Coomassie Brilliant Blue (R-250) (Sigma-Aldrich, Missouri, USA) 10% (v/v) acetic acid (Merck, Darmstadt, Germany)
Destaining:	10% (v/v) acetic acid (Merck, Darmstadt, Germany) 1% (v/v) glycerol (Sigma-Aldrich, Missouri, USA)

EXTRACTION OF RNA

5M GITC:	Guadinine thiocyanate (295.4 g) (Sigma-Aldrich, Missouri, USA) Sodium N-Lauroyl Sarcosine (2.5 g) (Sigma-Aldrich, Missouri, USA) β-mercapto ethanol (3.5ml) (Sigma-Aldrich, Missouri, USA) 12.5 M Sodium citrate pH 7.0 (Sigma-Aldrich, Missouri, USA) Tween80 (5 ml) (Sigma-Aldrich, Missouri, USA)

3.7. REFERENCE LIST

1. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and a. et**. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393**:537-544.
2. **Livak, K. J. and T. D. Schmittgen**. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**:402-408.
3. **Michael W Pfaffl**. 2004. Quantification strategies in real-time PCR, *In* S.A Bustin (ed.), A-Z of quantitative PCR. International University Line (IUL), La Jolla, CA, USA.
4. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**:e45.
5. **Streicher, E. M., R. M. Warren, C. Kewley, J. Simpson, N. Rastogi, C. Sola, G. D. van der Spuy, P. D. van Helden, and T. C. Victor**. 2004. Genotypic and phenotypic characterization of drug-resistant Mycobacterium tuberculosis isolates from rural districts of the Western Cape Province of South Africa. J.Clin.Microbiol. **42**:891-894.

CHAPTER 4

RESULTS

4.1. PROTEOMICS

Three comparative experiments using PD-Quest Advanced 2-D analysis software (Version 8.0) (Bio-Rad Laboratories, Hercules, CA 94547) were done to determine the global membrane protein expression profile of *M. tuberculosis* clinical strains after RIF exposure at the critical concentration (2µg/ml) for 24 hours. Biological and technical replicas were done for each experiment. In experiment 1 (Figure 4.1) and 2 (Figure 4.2), RIF exposed strains with a high MIC (R451) and intermediate MIC (R439) was compared to the RIF unexposed control group of R451 and R439 respectively. In the third experiment (Figure 4.3) a comparative analysis was done between the High MIC (R451) and intermediate MIC (R439) strains when exposed to RIF. Figures 4.1, 4.2, 4.3 show the electrophoretic profiles of proteins seperated with 2-DE.

All proteins were separated on a 12% polyacrylamide gel with a pI range of 4-7 and a molecular weight of 10-75kDa. Statistical analysis was done by using a PD Quest (Version 8.0) (Bio-Rad Laboratories, Hercules, CA 94547) integrated statistical analysis tool (T-test) with a confidence interval of 95% (p-value of <0.05). Arbitrary density values were assigned by PD Quest to each differentially expressed spot. Differentially expressed spots between the experimental conditions were expressed as a ratio of the test versus the control when one of the two was set as 1.

4.1.1. Experiment 1: High MIC strain (R451): RIF exposed vs. unexposed

PD Quest analysis identified 220 protein spots of which 86 were significantly differentially expressed ($p=0.05$) between the RIF exposed and unexposed conditions. From the 86 spots (Table 4.1), 64 were unique to RIF exposed gels, 6 spots were unique for the unexposed gels and 16 were present in both RIF exposed and unexposed gels. From these 16 spots, 7 were up regulated in the unexposed condition and 9 were up regulated in the exposed condition. These 86 protein spots were also visually inspected to refine the analysis before 20 spots were excised from the gel and submitted for mass spectrometry.

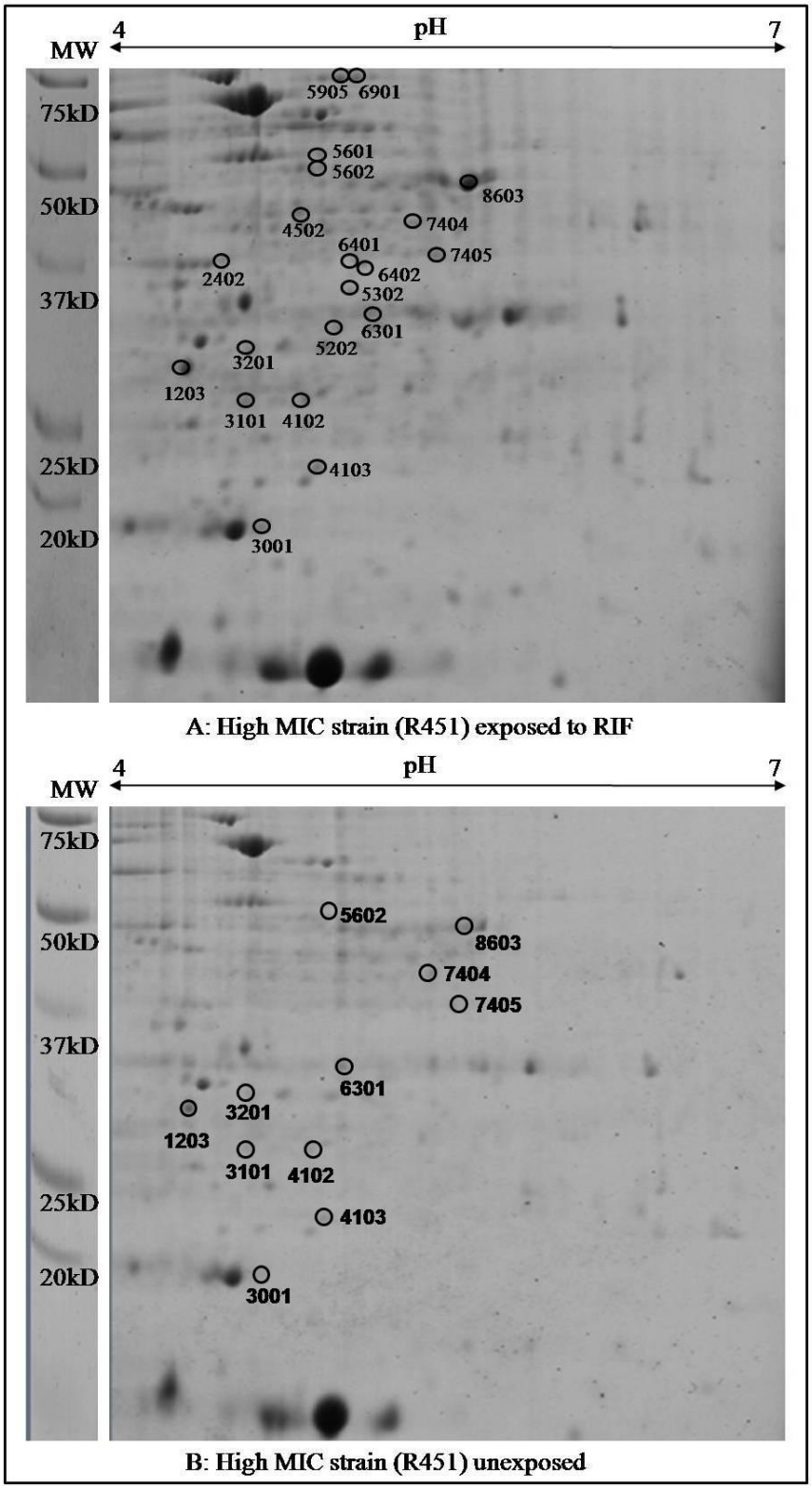

Figure 4.1: Two dimensional electrophoresis patterns of membrane proteins from a *M. tuberculosis* high RIF MIC DRF 150 strain (R451). (A) Exposed to 2µg/ml Rifampicin and (B) unexposed. Proteins were stained with Coomassie brilliant blue. Molecular mass range (in kilodaltons) and pI range (between 4 and 7) are indicated. Circled spots were excised and submitted for mass spectrometry analysis

Table 4.1: Differentially expressed protein spots between RIF exposed and unexposed high MIC strain (R451)

Spots							
SSP nr	**Unexposed**	**RIF exposed**	**Ratio**	**SSP nr**	**Unexposed**	**RIF exposed**	**Ratio**
101	-	39.9	19.31	4604	-	13.5	6.52
401	-	15.9	7.67	4902	34.7	23.4	0.67
402	-	27.4	13.26	4905	-	8.5	4.09
403	10.2	-	0.07	5202	-	14.7	7.13
501	33.1	4.8	0.14	5301	-	16.2	7.83
502	-	24.5	11.88	5302	-	8.6	4.18
503	-	20.4	9.87	5402	-	10.5	5.08
504	26.8	19.6	0.73	5601	-	8.6	4.14
505	-	11	5.32	5602	-	9.9	4.81
1202	28.4	22.8	0.8	5902	-	18.4	8.91
1203	68	116.7	1.72	5905	-	27.1	13.13
1301	-	23.2	11.22	6101	-	8.4	4.08
1501	21.3	45.3	2.13	6202	-	16.1	7.79
1502	34.2	55.8	1.63	6203	-	11.3	5.48
1701	-	8.3	4.02	6301	-	44.2	21.41
2001	135.8	107	0.79	6401	-	7.1	3.46
2002	-	52.2	25.25	6402	-	9	4.38
2005	139	-	0.01	6901	-	23.7	11.45
2401	18.1	25.3	1.39	6902	-	11.9	5.74
2402	-	17.7	8.55	7001	23.7	32.7	1.38
2404	-	48.9	23.67	7301	-	8.7	4.22
2501	-	14.3	6.93	7401	-	6.2	2.99
2601	19.7	26.4	1.34	7402	-	10.5	5.08
2702	-	12.8	6.2	7403	-	12.1	5.85
2901	-	31.7	15.34	7404	-	22.3	10.77
2902	-	7.3	3.54	7405	-	28.3	13.69
3001	-	42.4	20.52	7501	-	16.8	8.13
3101	-	30.3	14.67	7503	-	28.3	13.68
3201	-	19.8	9.61	7602	-	11.8	5.71
3601	66.2	54.9	0.83	7606	-	44	21.32
3603	60.4	77.2	1.28	7608	-	22	10.66
3701	-	12.9	6.26	7901	-	12.4	6.01
3703	-	26.9	13	8104	-	28	13.55
3906	149.6	-	0	8205	59.4	-	0.01
4101	27	30.6	1.13	8305	-	51.1	24.75
4102	-	22.6	10.93	8307	-	22.4	10.82
4103	-	26.7	12.93	8310	43.5	121.1	2.78
4302	24.9	-	0.03	8501	-	13.9	6.74
4401	22.3	18.9	0.85	8601	-	88.8	42.99
4402	-	11.6	5.6	8603	-	194.2	94.03
4502	-	18.3	8.86	9102	-	30.7	14.85
4601	-	15.3	7.42	9103	-	17.7	8.55
4602	-	11.3	5.46	9105	29	-	0.03

Legend to table 4.1:
SSP number = number assigned to protein spots by PD Quest software
Arbitrary density values were assigned by PD Quest software to each differentially expressed spot
- = absent protein spot, highlighted spots were submitted for mass spectrometry analysis

MASS SPECTROMETRY ANALYSIS

In total 21 proteins were identified from the 20 spots submitted for mass spectrometry. Spots 3101 and 6301 were each identified as a mixture of two unique proteins. No positive identification for spot 2402 could be made due to insignificant MOWSE scores. Table 4.2 depict the electrophoretic characteristics and matched peptides of the identified spots.

Table 4.2: Electrophoretic characteristics and matched peptides of identified spots from Experiment 1

Spot nr	Predicted MW (kDa)	pI	Matched peptides Position start/end sequence	MOWSE Score *	Queries Matched	Coverage % ᵠ
1203	28064	4.66	10-72/ QVPDTWSERKLTDGDFTLDREAADAVLDEINERAVE EALQIREKEAADGIE GSVTVLTAGP ER 88-110/ AVHLKDDGMHGSDVIQTGWALAR 161-197 ITGERETDEGVFTLEATLPAVISVNEKINEPRFPSFK 241-264/ TAGEKVTDEGEGGNQIVQYLVAQK	192	15	55
2402	No positive Identification					
3001	20429	4.73	1-8/ MTEYEGPK 47-100/ HFYSQAVEERNHAMMLVQHLLDRDLRVEIPGVDT VRNQFDRPREALALALDQER 149-171/ AGANLFELENFVAREVDVAPAASGAPHAAGGRL	145	10	52
3101[§]	56561	4.85	1-12/ AKTIAYDEEA RR 17-26/ GLNALADAVK 41-56/ KWGAPTITNDGVSIAK 78-99/ KTDDVAGDGTTTATVLAQALVR 195-207/ GYISGYFVTDPER 320-342/ VVVTKDETTIVEGAGDTDAIA GR 348-361/ QEIENSDSDYDREK 378-397/ AGAATEVELKERKHRIEDAVR 449-464/ QI/AFNSGLEPGVVAEK	205	23	27
	27529	4.84	2-29/ MADPGPFVAD LRAESDDLDALVAHLPADR 104-117/ GRLHEELLAVPDGR 172-255/ DYAFIVNNLTPPAEPFLVELRGPSGDTWSWGPSDA AQRVTGSAEDFCFLVTQRRALSTLDVNAVGEDAQRWLTIA QAFAGPPGR			49
3201	30046	4.97	55-63/ DIVWEQVQR 102-110/ RDIGGVMVR 117-125/ GDRHVIAAR 189-226/ VYAEIVGNPTGWVEIVASQRHPG GTTTQTDAAAG VLDSK 236-281/ RVGGDLYGSFLPGTQQNLERALDGLLELLPAGAW LDHTSDHAQASSR	59	8	39
4102	27256	4.93	2-10/ TYETILVER 134-145/ LGVLPGM GGSQR 155-163/ AMDLILTGR 179-205/ VVPADDLLTEARATATTISQMSASAAR 214-227/ AFESSLSEGLLYER 200-257/ AAPQFTHR	66	7	30
4103	20113	5.02	1-13/ MRVLLLGPPGAGK 24-36/ LGIPQISTGELFR 52-88/ LDAGDLVPSDLTNELVDDRLNNPDAANGFILDGYPR 95-123/ ALHEMLERRGTDIDAVLEFRVSEEVLLER 128-140/ GRADDTDDVILNR 143-156/ VYRDETAPLLEYYR 161-174/ TVDAVGTMDEVFAR	114	13	73

4502	42628	4.92	2-11/ PEAVIVSTAR 71-88/ VVAVALGYDFLPGTTVNR 105-122/AGEGDAFISAGVETVSR 126-160/ GNSDSWPDTKNPLFDGAQERSAAAAAGADEWHD PR 188-239/ EEQDRWGVRSQNRAEEAIKNGFF EREITPVTLPDG TTVSTDDGPRPGTTYEK 276-286/ AKELGLTPLAR 315-390/AGMAITDIDLVEINEAFAVQVLGSAR	103	11	41
5202	30294	5.17	70-86/ V GTFNPAALLH GSQGIR 152-182/ GERPAAPEFPDRHPDARID MLTREDQALIYR 220-239/ ALVAELGGGVAANITSIAAR 266-278/TEVAGSDGAEAR	78	5	27
5302	32910	5.12	1-15/ MRPALSDYQHVASGK 18-51/ EIYRVDDEHLLLVASDRISAYDYVLDSTIPDKGR 104-119/ GYLTGSGLLDYQATGK 135-145/ FATPLFTPATK 176-274/TLQTYVQAADHALTRGIIIADTKFE FGIDRHGNLLL ADEIFTPDSSRYWPADDYRAGVVQTSFDKQFVRSWLTGSE SGWDRGSDRPPPPLPEHIVEATR 277-297/ YINAYERISELKFDDWIGPGA	160	17	65
5601	43345	4.93	1-9/ MELLRGALR 77-86/ FGDVLPFLVK 115-149/ IPVSSPVRNYRDTSKPELLVALQPFEALAGFR 160-183/ ALAVSDLDPFIDLLSEGSDADGLR 231-283/ YPGDAGVLAALLLNRISLAPGEAIFLPAGNLHAYV RGFGVEVMANSDNVLR 290-307/ HVDVPELLRVLDFAPTPK 379-392/ GTAAWVAADDGPIR	96	12	39
5602	56561	4.85	1-12/ AKTIAYDEEARR 33-56/ GRNVVLEKKWGAPTITNDGVSIAK 78-99/ KTDDVAGDGTTTATVLAQALVR 104-115/ NVAAGANPLGLK 195-207/GYISGYFVTDPER 320-342/ VVVTKDETTIVEGAGDTDAIAGR 348-361/ QEIENSDSDYDREK 378-398/ AGAATEVELKERKHRIEDAVR 428-440/ LEGDEATGANIVK	94	15	28
5905	59252	5.03	93-121/ RTGEVLSVPVGDGFLGRVVNPLGQPIDGR 130-154/ RALELQAPSVVHRQGVKEPLQTGIK 168-175/ QLIIGDRK 295-307/ EAYPGDVFYLHSR 315-333/ LSDDLGGGSLTGLPIIETK 395-418/ LDLSQYRELEAFAAFASDLDAASK 466-491/ RFETELLDHMRASEEEILTEIRDSQK	120	14	26
6301 [§]	34638	5.11	2-21/ TSQGDTTSGTQLKPPVEAVR 113-122/ AMFDEVDSPR 128-178/ IQGWEEFDEPVDRIVSLGAFEHFADGAGDAGFERY DTFFKKFYNLTPDDGR 209-219/ FILTEIFPGGR 223-237/ ISQVDYYSSNAGWK 270-282/ GQETYDIYMHYLR	123	17	39
	28737		2-11/ ANFTAADVKR 25-42/ NALAETDGDFDKAVEALR 128-135/ IGEKLELR 137-150/ VAIFDGTVEAYLHR 167-184/ GDDAAAAHAVALQIAALR 187-202/ YLSRDDVPEDIVASER 203-210/ RIAEETAR			
6402	32697	5.2	69-77/ GDPYADRPR 84-96/ VGADAGAGEFVVLR 144-159/ RITDATNAIDPLSAIK 162-176/ LAGYGAGDTDLAWSR 206-220/ TEPALDVLAGWLASR2 228-270/ AVGELKVELVRNSETIVLSRPQEGITATLTRTGKPD ALVPLAR	66	8	36

6901	59252	5.03	93-121/ RTGEVLSVPVGDGFLGRVVNPLGQPIDGR 130-151/ RALELQAPSVVHRQGVKEPLQTGIK 295-307/ EAYPGDVFYLHSR 315-333/ LSDDLGGGSLTGLPIIETK 465-491/ RFETELLDHMRASEEEILTEIRDSQK	74	11	20
7404	40815	5.2	59-63/ DAKQELIDSLEEAVR 75-99/ ALIATGEQVLVNEHLIGPPPATVIR 116-132/ RPTYVFAAVDHTGADVK 187-215/ LVDAADPEVVFVSGEVRSRTDLLSTLPQR 220-247/ VSQLHAGPRKSALDEEEIWDLTSAEFTR 250-266/ YAEITNVAQQFEAEIGR 329-347/ AD EALPFAAIAVGAALVR 351-368/ IAPLDGVGALLRYAATNR	103	11	44
6401	35538	4.95	3-23/ TPATVVAGVDLGDAVFAAAVR 29-38/ VEQLMDTELR 164-177/ GTSENVDSIEQYLK 93-207/ LGGMFSGATDEQVER 268-281/ ALLNGPVDDDAEVR 296-243/ AKDVLAQYAAQARHELALLPDVPGRRALAALVD YTVSR	80	9	33
7405	36536	5.28	21-57/ APSLHNSQPWRWIAEDHTVALFLDKDRVLYATDHSG R 72-85/ VAMAAAGTTANVER 112-191/ LRADAILLRRTDRLPFAEPPDWDLVESQLR 151-167/ IDVIADDMRPELAAASK 215-245/ DFPVVANTDRRPEFGHDRSKVLVLSTYDNER 283-326/ DLVAALIGQPATPQALVRVGLAPEMEEPPPATPRR PIDEVFHVR	166	17	52
8603	43566	5.28	9-25/ TKPHVNIGTIGH VDHGK 35-59/ VLHDKF PDLNETKAFDQIDNAPEER 62-92/GITINIAHVYQTDKRHYAHVDAPGHADYIK 140-173/ ADAVDDEELLELVEMEVRELL AAQEFDEDAPVVR 236-282/GVINVNEEVEIVGIRPSTTKTTVTG VEMFRKLLDQGQAGDNVGLLLR 365-376/ LIQPVAMDEGLR	95	12	41

Legend for table 4.2:
***** According to Mascot results, Protein MOWSE scores greater than 52 were considered as signifcant.
§ Spots were found to be a mixture of two or more proteins, each described in a row of the table
Ψ Percentage of protein sequence covered with identified peptides

4.1.2. Experiment 2: Intermediate MIC strain (R439): RIF exposed vs. unexposed

PD Quest analysis identified 231protein spots of which 53 were differentially expressed (p=0.05) between the RIF exposed and unexposed conditions. From the 53 spots (Table 4.3), 11 were unique for the exposed condition, 20 were unique for the unexposed condition and 22 were present in both the RIF exposed and unexposed gels. From these 22 spots, 5 were up regulated in the unexposed condition and 17 were up regulated in the exposed condition. When visually inspected, none of the spots described in Table 4.3 were observed as differentially expressed and therefore none were picked and excised for mass spectrometry. These results could not be due to experimental error since cultivation, RIF exposure, protein extractions and 2-DE gels were done in biological and technical replicas.

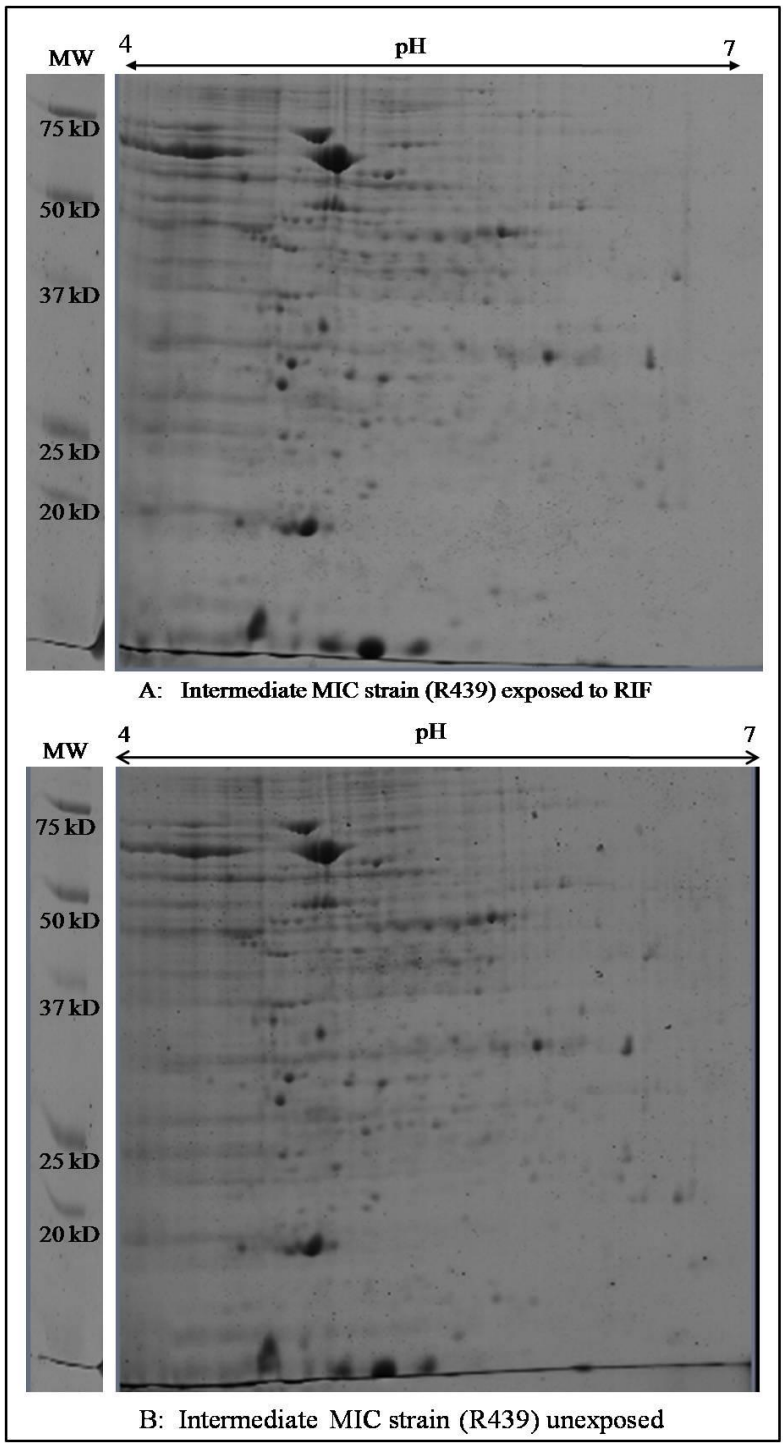

Figure 4.2: Two dimensional electrophoresis patterns of membrane proteins from a *M. tuberculosis* intermediate RIF MIC DRF 150 strain (R439). (A) Exposed to 2µg/ml RIF and (B) unexposed. Proteins were stained with Coomassie brilliant blue. Molecular mass range (in kilo daltons) and pI range (between 4 and 7) are indicated.

Table 4.3: Differentially expressed protein spots between Intermediate MIC strain (R439) RIF exposed and unexposed.

Spots							
SSP nr	Unexposed	RIF exposed	Ratio	SSP nr	Unexposed	RIF exposed	Ratio
1102	58.1	115.1	1.98	4506	28.9	17.8	0.61
1202	12.4	19.4	1.56	5003	328.3	-	0.01
2101	42.9	315.5	7.35	5201	39.2	56.6	1.44
2102	175.1	-	0.02	5503	14.1	10.7	0.76
2104	432.9	216.4	0.5	5505	67.2	-	0.05
2203	17.8	-	0.18	5506	-	14.5	15.38
2205	17.4	32.3	1.85	6201	24.6	-	0.13
2206	-	34.5	36.58	6203	18	28.4	1.58
2207	-	8.9	9.43	6301	18.8	-	0.17
2405	18.4	39	2.12	6308	-	9.2	9.76
3003	-	74.4	78.8	6404	15	-	0.22
3101	93.4	118.9	1.27	6504	35.6	-	0.09
3103	131.8	193.4	1.47	7104	29.9	-	0.11
3106	10.3	-	0.32	7106	-	15	15.92
3205	13.4	19.9	1.49	7202	25.8	37.8	1.46
3302	30	-	0.11	7406	10.4	11.7	1.13
3403	18.8	32	1.7	7501	92.1	56.8	0.62
3501	20.4	25.2	1.23	7507	132.1	67.9	0.51
3702	81.8	122.1	1.49	8101	25.8	36.3	1.41
3705	-	69.5	73.7	8201	41.1	-	0.08
4102	532.8	457.1	0.86	8202	11.8	-	0.28
4106	-	346.2	366.87	8204	22	-	0.15
4107	-	51.8	54.93	8401	9.3	-	0.35
4108	-	38.8	41.12	9101	18.8	-	0.17
4202	76.9	106.3	1.38	9106	43.4	-	0.08
4303	9.4	-	0.35	9112	-	31.8	33.66
4401	12.3	-	0.27	9202	26.5	-	0.12

Legend to Table 4.3
SSP number = number assigned to protein spots by PD Quest software, - = absent protein spot
Arbitrary density values were assigned by PD Quest software to each differentially expressed spot

4.1.3. Experiment 3: High MIC strain (R451) RIF exposed vs. Intermediate MIC strain (R439) RIF exposed

PD-Quest analysis identified 283 protein spots of which 116 were differentially expressed (p=0.05) between the two strains exposed to RIF. From the 116 spots (Table 4.5), 60 were unique for the high MIC strain, 16 were unique for the intermediate MIC strain and 40 were present in both strains when exposed to RIF. From these 40 spots, when compared to the intermediated MIC strain, 19 spots were more

expressed in the high MIC strain and 21 spots were less expressed in the high MIC strain. These 116 spots were also visually inspected to refine the analysis before 20 spots were excised from the gels and submitted for mass spectrometry. Five (Table 4.5) of these 20 spots were excised based on differences observed during visual inspection of the gels.

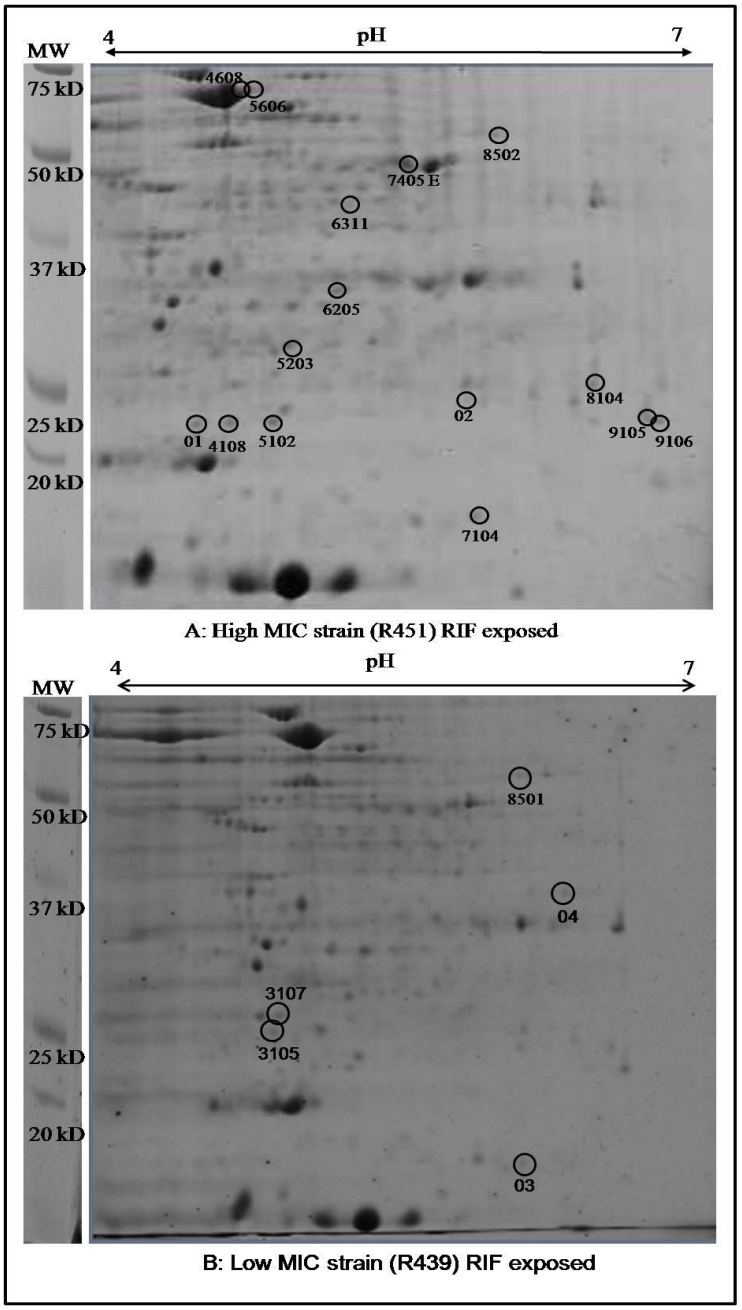

Figure 4.3: Two dimensional electrophoresis patterns of membrane proteins from *M. tuberculosis* DRF 150 RIF resistant strains. (A) High RIF MIC strain (R451) exposed to 2µg/ml Rifampicin and (B) Intermediate RIF MIC strain (R439) exposed to 2µg/ml. Proteins were stained with Coomassie brilliant blue. Molecular mass range (in kilodaltons) and pI range (between 4 and 7) are indicated. Circled spots were submitted for mass spectrometry analysis

Table 4.4: Differentially expressed protein spots between high MIC strain (R451) exposed to RIF and Intermediate MIC strain (R439) exposed to RIF

Spots							
SSP	Low MIC	High MIC	Ratio	SSP	Low MIC	High MIC	Ratio
2002		27	57.73	5606		13.1	28.05
2303	6.4		0.07	6201	63.1	29.8	0.47
2304		20.7	44.44	6203	14.8	18.9	1.27
2305		18.8	40.34	6205		32	68.48
2403	19.8	14.8	0.75	6206		9.3	19.96
2409		1.4	3.06	6207		7.6	16.31
3001		52.9	113.33	6208		5	10.62
3104	123.4	91	0.74	6209		3.4	7.31
3105	16.2		0.03	6302	35.7	8.5	0.24
3107	16.1		0.03	6303		13.3	28.44
3108		12.6	27	6304		12	25.63
3201	24.2	17.5	0.72	6305		6.1	13.13
3203	89.8	94.1	1.05	6306		6.7	14.43
3204	101.4	80.4	0.79	6307		5.6	12.02
3306		19.2	41.19	6308		4.5	9.73
3307	25	51.5	2.06	6309		3.7	8
3401	12.7	43.7	3.45	6311		3.1	6.69
3501	14.4		0.03	6315	5.8		0.08
3502	20.3		0.02	6401	26.7	9.4	0.35
3503	16.1		0.03	6402	13.2	18.9	1.43
3504	19.8		0.02	6404	31	19.4	0.63
3603	10	4.1	0.41	6405	46.4	30	0.65
3604	10.4		0.05	6406		5.9	12.53
3605	5.2		0.09	6503		7.7	16.46
3606		26.5	56.85	6602	51.3	71.4	1.39
3705		5.5	11.83	6604	22.1	6.6	0.3
4001	67.2		0.01	6605	24.4	52.3	2.14
4108	8.1	19.6	2.43	6606	30.7	40.5	1.32
4202	21.1		0.02	6608		15	32.18
4205		22.8	48.77	7102	29.4		0.02
4206		16	34.35	7103		27	57.75
4302	8.7	12	1.37	7104		15.6	33.42
4304	5.3	19.9	3.78	7201	30.6	43.7	1.43
4306		14.6	31.2	7206	34		0.01
4308		7	14.91	7208	115.9	177.4	1.53
4503	15.5		0.03	7209		60.3	129.2
4504	70.8	27.3	0.39	7210		29.3	62.69
4505	51.3	50.2	0.98	7211		5.2	11.07
4507	13		0.04	7212		97.7	209.24
4508	82.5	72.2	0.88	7402	55.3	18.3	0.33
4509		6.7	14.25	7405		71.7	153.63
4510		10.7	23.01	7406	14.1	56	3.98
4603	37.9	7.1	0.19	7407	79.8	146.7	1.84
4606	2.1	3.8	1.82	7409		19.5	41.87
4607		288.2	617.26	7410		15	32.1
4608		24.8	53.03	7411		13.2	28.36

4701	132.9	88	0.66	7412		1.8	3.77
5102	15.1	26.2	1.74	7413		15.6	33.49
5103		13.7	29.35	7501		3.4	7.29
5203		18.8	40.2	7601		8.5	18.12
5301	3.6	13.4	3.76	8103		23.5	50.33
5501	40.7	12.3	0.3	8104		18.3	39.26
5502	14.2	7.5	0.53	8203		12	25.61
5503	9	16.9	1.89	8302		18	38.53
5505	11.4	7.2	0.63	8303		14.3	30.72
5506		6	12.75	8502	14.9	3	0.2
5507		2.3	4.93	9105		17.6	37.61
5604	26.1	53.6	2.05	9106		14	30.09
5605		78.8	168.88				

Legend for Table 4.4:
SSP number = number assigned to protein spots by PD Quest software, - = absent protein spot, highlighted spots were send for mass spectrometry analysis
Arbitrary density values were assigned by PD Quest software to each differentially expressed spot

Table 4.5: Differential expressed protein spots between high MIC strain (R451) exposed to RIF and Intermediate MIC strain (R439) exposed to RIF observed with visual inspection.

Spot nr	High MIC strain RIF exposed	Intermediate MIC strain RIF exposed	Differential expression
01	Present	Present	Up regulated in High MIC
02	Present	Absent	
03	Absent	Present	
04	Absent	Present	
4608	Present	Present	Up regulated in High MIC

MASS SPECTROMETRY ANALYSIS

In total 20 proteins were identified from the 20 spots submitted for mass spectrometry. Spots 4608, 5203, 6205 and 7405 were each identified as a mixture of two or more unique proteins. No positive identification for spot 02, 3105, 6311, 7104 and 9105 could be made due to insignificant MOWSE scores. Table 4.6 depict the electrophoretic characteristics and matched peptides of the identified spots.

Table 4.6: Electrophoretic characteristics and matched peptides of identified spots from Experiment 3

Spot nr	Predicted MW	pI	Matched peptides Position start/end sequence	MOWSE Score*	Queries Matched	Coverage %[Ψ]
1	23525	4.99	14-40/ YILPSFIEHSSFGVKESNPYNKLFEER 132-159/ VLIHQPSLSGVIQGQFSDLEIQAAEIER 162-180/ TLMETTLARHTGKDAGVIR 188-207/ ILTAEEAKDYGIIDTVLEYR	86	7	43
02	No positive Identification					
03	17896	5.49	5- 25/ VGDTVVYPHHGAALVEAIETR 39-87/ VAQGDLTVRVPAENAEYVGVRDVVGQEGLDKVFQVLRAP HTEEPTNWSR 150- 162/ AETILDEVLAAAS	84	8	51
04	365522	5.49	2-17/ PIATPEVYAEMLGQAK 40-85/ GFADAGSDGIIQFSTGGAEFGSGLGVKDMVTGAVALAEFTH VIAAK 100-116/ DKLDSYVRPLLAISAQR 156-179/ IILEI EIGVVGGEEDGVANEINEK 202-267/ YLLAATFGNVHGVYKPGNVKLRPDILAQGQQVAAAKLG PADAKPFDFVFHGGSGSLKSEIEEALR 319-340/ KAEASMSQRVVQACNDLHCAGK	87	14	55
3105	No positive Identification					
3107	30286	4.65	64-86/YPGGVVMAGDRRSTQGNMISGR 90-146/ KVYITDDYTATGIAGTAAVAVEFARLYAVELEHYEKLEGV PLTFAGK 177-204/ IVSFDAAGGWNIEEEGYQAVGSGSLFAK 209-266/ KLYSQVTDGDSGLRVAVEALYDAADDDSATGGPDLVRG IFPTAVIIDADGAVDVPESR	126	14	53
4108	23525	4.99	14-38/ YILPSFIEHSSFGVK 132-159/ VLIHQPSLSGVIQGQFSDLEIQAAEIER 162-170/ TLMETTLAR 188-207/ ILTAEEAKDYGIIDTVLEYR	59	5	33
4608[§]	56561	4.85	3-12/ TIAYDEEARR 41-66/ KWGAPTITNDGVSIAKEIELEDPYEK 78-99/ KTDDVAGDGTTTATVLAQALVR 104-166/ NVAAGANPLGLKR 140-223/EQIAATAAISAGDQSIGDLIAEAMDKVGNEGVITVEESNTF GLQLELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSSK 229-236/ DLLPLLEK 320-342/ VVVTKDETTIVEGAGDTDAIAGR 348- 361/ QEIENSDSDYDREK 391-398/ HRIEDAVR 402-440/ AAVEEGIVA GGGVTLLQAAPTLDELKLEGDEATGANIVK 449-464/ QIAFNSGLEPGVVAEK 467-494/ NLPAGHGLNAQTGVYEDLLAAGVADPVK 497-523/ SALQNAASIAGLFLTTEAVVADKPEK	313	41	58
	62203	4.91	20-37/ HTLSQLRLHELLVEVQDR 70-82/ AIVHSATSLVDAR 95-110/ VLHFVYEGIDEETVRR 117-132/ GLGVIGLLIEDPKP LR 212-237/ QSWIEATRDIATELLSGTEPATVFR 287-305/TIPVAGAVLREVFVNGIPR 310-330/VDLEGLDELADAGPALLLPLR 378-387/ MRELDVLTDR 393-401/ DLHDHVIQR 425-455/ LSDVVDDLQDVIQEIRTTIYDLHGASQGITR 460-498/ IDAAVAQFADSGLRTSVQFVGPLSVVDSALADQAEAVVR 517-547/ VKVDDDLCIEVTDNGRGLPDEFTGSGLTNLR			46

			550-571/ AEQAGGEFTLASVPGASGTVLR			
5102	23525	4.99	14-28/ YILPSFIEHSSFGVK 132-159/ VLIHQPSLSGVIQGQFSDLEIQAAEIER 162-170/ TLMETTLAR 188-207/ ILTAEEAKDYGIIDTVLEYR	57	5	33
5203 §	25964	5.21	26-51/ EIADALLAALERRHEVADAIVEAANK 159-201/ RVDDEEAAWFVAVDSGVKVGMVFGELVHGEVDVRIWIH PDHRK 203-211/ GYGTAALRK 229-232/ APAAQPAQPGSAGR	88	18	38
	28879	5.08	32-41/ EASVSLSEIK 70-97/ RASPSAGALATIADPAKLAQAYQDGGAR 107-119/ RFQGSLDDLDAVR 129-142/KDFVVQPYQIHEAR 168-187/ TESLGMIALVEVHTEQEADR			30
5606	56561	4.85	3-12/ TIAYDEEARR 17-26/ GLNALADAVK 41-66/ KWGAPTITNDGVSIAKEIEL EDPYEK 78-99/ KTD DVAGDGTTTA TVLAQALVR 104-115/NVAAGAN PLGLK 166-191/VGNEG VITVEESNTF GLQLELTEGM R 195-223/GYISGYFVTDPERQEA VLEDPYILLV SSK 320-342/V VVTKDETTIV EGAGDTDAIA GR 348-361/QEIENSDSDYDRE K 390-398/HRIEDAVR 449-464/ QIAFNSGLEPGV VAEK 367-494/ NLPA GHGLNAQTGV YEDLLAAGVA DPVK 498-523/ SALQNAASIAGLF LTTEAVVADK PEK	158	20	46
6205 §	34284	5.10	2-14/ SAMRTHDDT WDIK 69-84/ AA AIDAETAAIV AYLR 91-119/ TNFFDTYFASAVAAGIRQVV ILASGLDSR 123-138/ LDWPAGTI VYEIDQPK 160-174/E VPADLRQDWP AALR 203-229/ LFTQVGAV SVAGSRIAAE TAPVHGEER 263-275/ ASVADWLT DHGWR	64	19	41
	30277	5.17	70-113/ V GTFNPAALLH GSQGIRLHAP LPAAGKLSVVTEVADIQDKG EGK 152-182/ GERPAAPEF PDRHPDARID MPTREDQALI YR 220-257/ A LVAELGGGVA ANITSIAARF TKPVFPGETLSTVIWR 266-277/ TEVAG SDGAEAR			42
6311	No positive Identification					
7104	No positive Identification					
7405 §	43566	5.28	35-59/ VLHDKF PDLNETKAFDQIDNAPEER 62-92/ GITINIAHV EYQTDKRHYA HVDAPGHADY IK 127-173/ QVGV PYILVALNKA DAVDDEELLELVEMEVRELL AAQEFDEDAP VVR 185-225/ WVASV EELMNAVDESIPDPVRETDK PFLMPVEDVF TITGR 236- 282/ GVINV NEEVEIVGIRPSTTKTTVTG VEMFRKLLDQ GQAGDNVGLL LR 292-317/ GQVVTKPGTTTPHTEFEGQ VYILSK 322-336/ HTPFFNNYR PQFYFR 365-376/ LIQPVA MDEGLR	296	40	61
	47948	5.36	1-11/ MADTDDTATL R 57-70/ SSIT YIDGDAGILR 73-82/ GYPIDQLA EKS 113-122/ HTMLHEDL KR 182-220/ SVGQPFLYP DNSLTLVENF LRLTFGFPAE PYQADPEVVR 345-359/ ELEEAALTDDYFIER 360-374/ LYPNVDFYTG LIYR 392-400/ LPGWIAHWR 414-431/ QIYTGY TERDYVTIDA R			32

	48776	5.4	83-97/ IAEQLEAQ ADVEAER 119-127/LE LGHESVR 221-233/ YLTVPAEDA TPR 237-254/ LIERL VSGKVGAPTLEVLR 333-372/ ADSTVNP VVVALLSHTVELLRGQAVEE AVLFLAEVAV AR			23
8104	23279	5.62	4-15/ VFLVDDH EVVRR 43-56/VPAARPDVAVLDVR 156-168/TLLGL LSEGLTNK 198-209/ TQAAVFATELKR	58	5	23
8501	49208	5.53	2-31/ THYDVVVLG AGPGGYVAAI R 56-64/ NAELVH IFTK 68-85/ AFG ISGEVTFDYG IAYDR 148-168/ LVPGTSLSANVVT YEEQILSR 192-205/ NYGVDVTIVEFLPR 439-448-/WD LTASELAR	66	6	20
8502	49208	5.53	2-35/ THYDVVVLG AGPGGYVAAI RAAQLGLSTA IVEPK 55-64/ NAELVH IFTK 148-168/ LVPGTSLSANVVT YEEQILSR 192-220/ NYGVDVTIV EFLPRALPNE DADVSKEIEK 252-298/DGVAQELKA EKVLQAIGFA PNVEGYGLDK AGVALTDRKA IGVDDYMR 385-196/ AHGVGD PSGFVK 429-438/ WD LTASELAR	72	13	35
9105	No positive Identification					
9106	14572	4.46	8-46/ FNH LVTVTDLETG DRAVCDRDQV AETIRAWFPD APLEVR 123-136/ MIGLGGGS PAEDER	56	4	38

Legend to Table 4.6:
***** According to Mascot results, Protein MOWSE scores greater than 52 were considered as significant.
§ Spots were found to be a mixture of two or more proteins, each described in a row of the table.
Ψ Percentage of protein sequence covered with identified peptides

PREDICTED FUNCTION OF IDENTIFIED PROTEINS

Predicted functions were assigned to the proteins identified from experiment 1 and 3 using the Tuberculist database (http://genolist.pasteur.fr/TubercuList) and then categorized according to their cellular processes (1). These processes include: (i) small molecule metabolism; (ii) cell processes; (iii) macro molecule metabolism and (iv) hypothetical proteins (Table 4.7). Proteins involved in small molecule metabolism could be further divided in the following processes: Energy metabolism (n=7); degradation (n=2); biosynthesis of cofactors, metabolic groups and carriers (n=2); lipid biosynthesis (n=1); central intermediate metabolism (n=1) or classified as purines, pyrimidines, necleosides and nucleotides (n=2). Macro molecule metabolism was further divided in two processes: Synthesis and modification of macro molecules (n=1) and degradation of macro molecules (n=1). Only one protein involved in cellular processes were identified. This protein functions as a chaperone or heat shock protein which prevents the misfolding of proteins and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions. In total, thirteen proteins were classified as hypothetical proteins.

Table 4.7: Functions of proteins identified through mass spectrometry analysis and bioinformatics and literature searches.

A. SMALL MOLECULE METABOLISM						
	Spot nr	**Exp nr** [#]	**Accession nr**	**Gene**	**Protein**	**Protein Function** [*]
Energy Metabolism	1203	1	Rv3029c	*fixA*	Electron transfer flavoprotein beta-subunit	Transfers electrons to main respiratory chain via ETF-ubiquinone oxidoreductase (ETF dehydrogenase)
	5905, 6901	1	Rv1308	*atpA*	ATP synthase alpha chain	Alpha chain (Regulatory unit) of FOF1 ATP synthase, which produces ATP from ADP in presence of proton gradient across membrane.
	6402	1	R1446c	*opcA*	Putative oxpp cycle protein	May be involved in functional assembly of glucose 6-phosphate dehydrogenase
	8501	3	Rv0462	*lpdC*	Dihydrolipoamide dehydrogenase lpd	Component of the alpha-ketoacid dehydrogenase complex
	04	3	Rv0363c	*fba*	Fructose bisphosphate aldolase	Involved in glycolysis (catalytic activity)
	7405	3	Rv0896	*gltA*	Citrate synthase GltA2	Involved in tricarboxylic acid cycle (Krebs cycle, catalytic activity)
	7405	3	RV1307	*atpH*	ATP synthase delta chain ATPH	FOF1 ATP synthase subunit delta. Either transmits conformational changes from CF(0) into CF(1) or is implicated in proton conduction
Degradation	4502, 4102	1	R1070c	*echA8*	Enoyl-coA hydratase	Could possibly oxidize fatty acids using specific components
	5601	1	R3255c	*manA*	Mannose-6-phosphate isomerase	Converts D-Mannose 6-Phosphate to D-fructose 6-phosphate
Biosynthesis of cofactors, metabolic groups and carriers	3001	1	Rv3841	*bfrB*	Possible bacterioferritin	Stores iron in a soluble, nontoxic, readily available form
	6401	1	Rv0562	*grcC1*	Polyprenyl-diphospate synthase	Possibly supplies polyprenyl diphosphate
Purines, pyrimindines, nucleosides and nucleotides	4103	1	Rv0733	*adk*	Adenylate kinase	Intracellular nucleotide metabolism. Acts as nucleoside mono-and di-phospate kinase in RNA and DNA biosynthesis
	5302	1	Rv0780	*purC*	Phosphribosylaminoimidazole-succinocarboxamide synthase	Involved in de novo purine biosynthesis

Lipid biosynthesis	6301	1	Rv0503c	cmaA2	Cyclopropane mycolic acid synthesis	Essential for cyclopropanation function. Transfers methylene group from S-adenosyl-L-methionine to cis double bond of unsaturated fatty acid chain, resulting in replacement of double bond with methylene bridge.
Central Intermediate metabolism	5203	3	Rv1611	trpC	Indole-3-glycerol phosphate synthase trpC	Involved in tryptophan biosynthesis pathway (catalytic activity)
B. MACRO MOLECULE METABLOLISM						
	Spot nr		**Accession nr**	**Gene**	**Protein**	**Protein Function**
Synthesis and modification of macromolecules	8603	1	Rv0685	*tuf*	Elongation factor TU TUF	Promotes the GTP-Dependent binding of aminoacyl-tRNA to A-site of ribosomes during protein biosynthesis
	7405	3				
	6301	1	Rv2889c	*tsf*	Elongation factor TU TSF	Associates with EF-TU.GDP complex and induces exchange of GDP to GTP, it remains bound to the aminoacyl-tRNA.EF-TU.GTP complex up to GTP hydrolysis stage on the ribosome.
Degradation of macromolecules	01, 4108, 5102	3	Rv2460c	clpP2	Endopeptidase Clp chain P2	Cleaves peptides in various proteins in a process that requires ATP hydrolysis
	3107	3	Rv2110c	prcB	Proteasome beta subunit PrcB	Protein degradation
C. **CELL PROCESSES**						
	Spot nr	**Exp nr**	**Accession nr**	**Gene**	**Protein**	**Protein Function**
Chaperones/ Heat shock	5602, 3101	1	Rv0440	*groEL 2*	Chaperonin 2	Prevents misfolding and promotes refolding and proper assembly of unfolded polypeptides generated under stress conditions
	4608, 5606	3				
D. HYPOTHETICAL PROTEINS						
	Spot nr	**Exp nr**	**Accession nr**	**Gene**	**Protein**	**Protein Function**
	3201	1	Rv3866			Unknown
	5202	1	R3389c			Unknown
	7404	1	Rv2629			Unknown
	3101	1	Rv0036c			Unknown
	7405	1	Rv2032			Unknown
	03	3	Rv3583c			Unknown
	4608	3	Rv3132c			Unknown
	5203	3	Rv0730			Unknown

	6205	3	Rv0146			Unknown
	6205	3	Rv3389c			Unknown
	8104	3	Rv3133c			Unknown
	8502	3	Rv0642			Unknown
	9106	3	Rv1573			Unknown

Legend to Table 4.7

Proteins identified in either experiment 1 or 3

* Protein function according to http://genolist.pasteur.fr/TubercuList and categorized according to (1).

Two genes, *atpA* (Spots 5905 and 6901) and *atpH* (spot 7405) indentified from two independent experiments were selected as candidates for subsequent transcriptomic and genomic analysis. These genes encodes for components involved in the production of energy that is essential for functions that includes membrane mediated metabolic processes such as efflux pumps. It has been shown that efflux mechanims regulate the intracellular concentration of RIF (Phd thesis, Gail E. Louw, 2009). Thus subsequent analysis focused on these two genes, *atpA* and *atpH*.

4.2. TRANSCRIPTOMICS

QRT-PCR was done to determine the expression levels of the two candidate genes in response to RIF. Both genes were significantly up regulated in both strains in the presence of RIF (Table 4.8; Figure 4.5). The *atpA* gene showed a 19.023 (p=0.001) and 11.633 (p=0.001) fold increase in the high RIF MIC strain (R451) and the intermediate RIF MIC strain (R439) respectively. Whereas the *atpH* gene showed a 7.554 (p=0.001) and 10.251 (p=0.005) fold increase in the high MIC strain (R451) and intermediate MIC strain (R439) respectively. Figure 4.4 is an example of a standard curve essay done for the reference gene, *16S*. For this assay a slope of -3.41 and coefficient correlation of -1 was achieved.

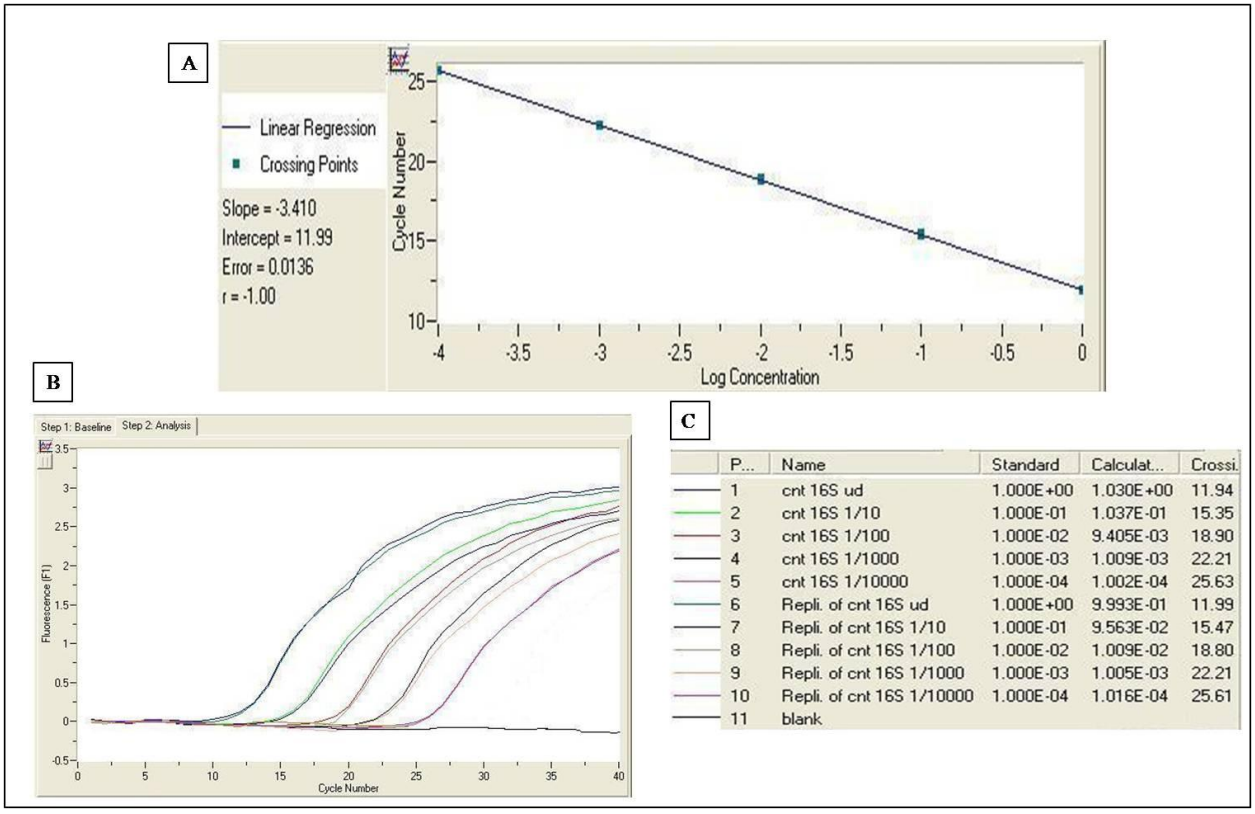

Figure 4.4: Standard curve of reference gene, *16S.*
A) The calculated C_T values were plotted against the log ng total cDNA of each starting quantity. Correlation coefficient (r) and slope values are indicated. Each sample was run in duplicate.
B) The calculated C_T values were plotted against measured Fluorescence (F1)
C) Duplicate C_T values for serial dilution of sample

Table 4.8: Differential gene expression profiles of candidate genes

Strain	*atpA*		*atpH*	
	Fold change *	**p-value ****	**Fold change ***	**p-value ****
R451 (high MIC)	19.023	0.001	7.554	0.001
R439 (intermediate MIC)	11.633	0.001	10.251	0.005

Legend to Table 4.8:
* Fold change after 24h exposure to 2µg/ml RIF
** p-value ≤ 0.05 were considered as significant

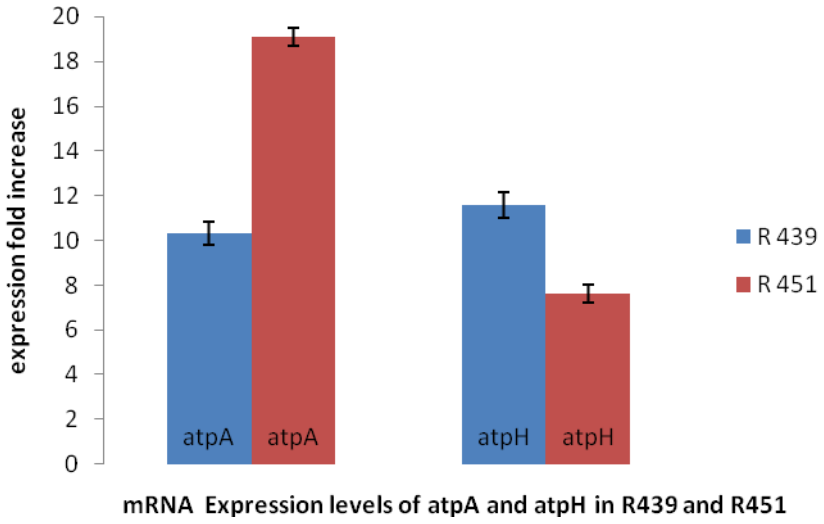

Figure 4.5: mRNA Expression levels of atpA and atpH in R439 (intermediate MIC strain) and R451 (high MIC strains) when exposed to 2µg/ml RIF.

4.3. GENOMICS

After screening 20 resistant and 15 susceptible clinical isolates from different strain families, no mutations could be found in either the coding regions of the genes (*atpA* or *atpH*) or 90bp upstream from these genes. However, sequencing revealed a synonymous polymorphism in the *atpH* gene at codon 91 which was specific for the LCC isolates (both drug resistant and susceptible isolates). It is therefore possible that this polymorphism can be used as a genetic marker for the LCC family. However, a larger study is needed to confirm this observation.

Additional bioinformatic analysis revealed that the *atpA* and *atpH* genes encode for two of the eight subunits that forms part the F_0F_1 ATP synthase enzyme (Table 4.9 and Figure 4.6). These genes are two of the largest in the cluster and each encodes for a catalytic subunit of FOF1 ATP synthase.

Table 4.9: Genes transcribing for the subunits of F_0F_1 ATP synthase

Gene	Accesion nr	Protein	Structural unit	Coding region start site	Coding region end site	Size (bp)
atpB	Rv1304	Subunit A	Membrane proton channel F0	1460244	1460996	752
atpE	Rv1305	Subunit C	Membrane proton channel F0	1461045	1461290	245
AtpF	Rv1306	Subunit B	Membrane proton channel	1461321	1461836	515
AtpH	Rv1307	Subunit delta	Catalytic subunit	1461843	1463183	1340
atpA	Rv1308	Subunit alpha	Catalytic subunit	1463228	1464877	1649
AtpG	Rv1309	Subunit gamma	Regulatory subunit	1464884	1465801	917
atpD	Rv1310	Subunit beta	Regulatory unit	1465841	1467301	1460
atpC	Rv1311	Subunit epsilon	Catalytic subunit	1467315	1467680	365

Figure 4.6: Gene orientation of gene cluster transcribing for subunits of F_0F_1 ATP synthase
* Nucleotide position of start and end site of gene cluster

SUMMARY OF RESULTS

Two candidate genes for transcriptomic and genomic analysis were identified through two independent proteomic experiments. Figure 4.7 summarizes the findings of this study.

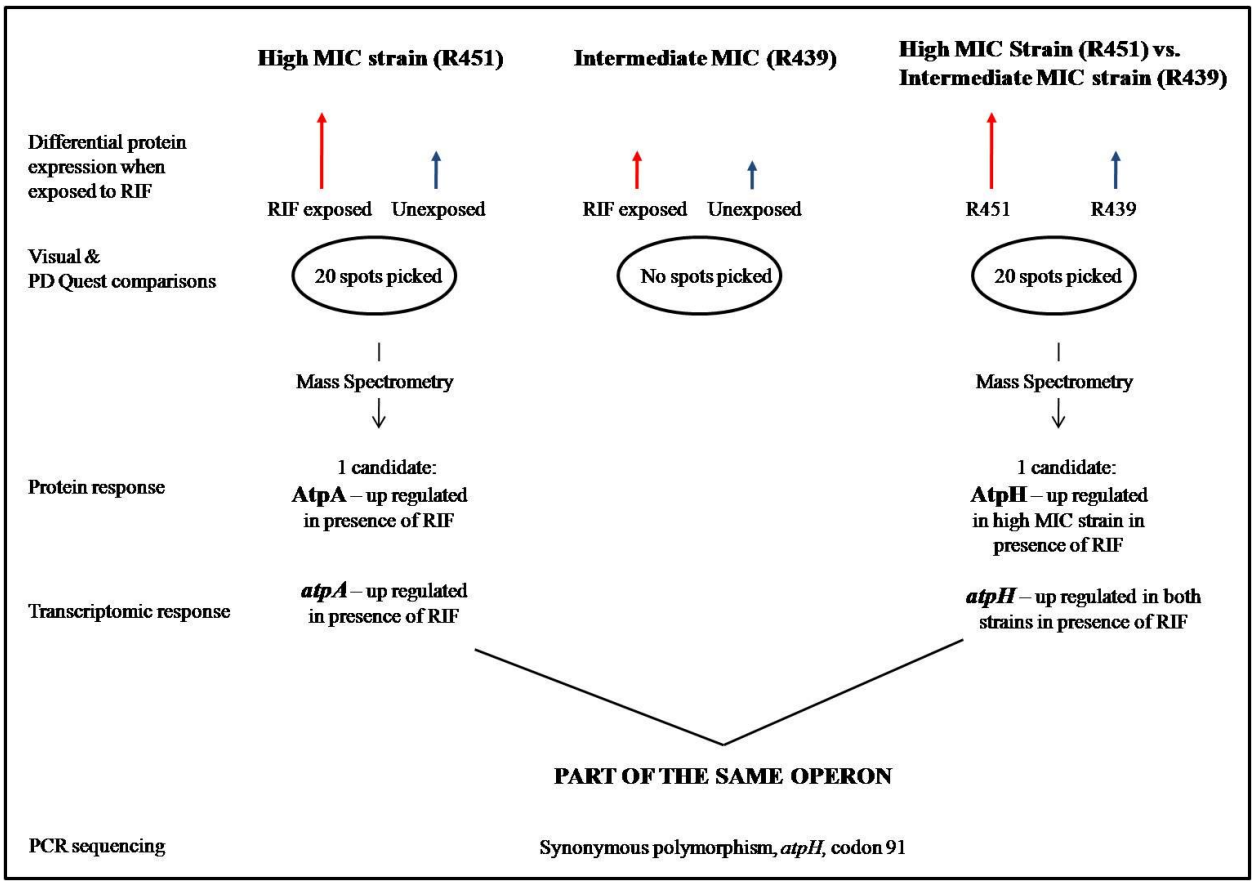

Figure 4.7: Summary of results from Proteomics. Coloured arrows indicate the differential expression of proteins in the presence of RIF.

4.4. REFERENCES

1. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and a. et**. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393**:537-544.

CHAPTER 5

DISCUSSION

This is the first study to demonstrate a proteomic response to RIF exposure in two RIF resistant clinical strains of *M. tuberculosis* which demonstrate different levels of RIF resistance despite having identical genetic backgrounds. Analysis of the 2D gels (visually inspected or by PD Quest software) showed that the abundance of membrane proteins increased in response to RIF. The PD Quest analytical method identified a total of 86 differentially expressed proteins in the high RIF resistant strain (R451). In contrast, the intermediate level RIF resistant strain (R439) showed less modification of protein expression after RIF exposure, with a total of 53 proteins being differentially expressed. However, the relative abundance of proteins in the unexposed and RIF exposed condition of the intermediate RIF resistant strain could not be accurately quantified by this software as has been previously described (7,15). Together these results suggest that the high level RIF resistant strain stimulates various different pathways in response to RIF exposure that might enable the strain to survive under higher concentrations of RIF.

Mass spectrometry of the most highly up regulated membrane proteins, identified proteins involved in a wide spectrum of cellular processes (Energy metabolism, degradation, biosynthesis of cofactors, metabolic groups and carriers, lipid biosynthesis, central intermediate metabolism, synthesis and modification of macromolecules, chaperone/heat shock proteins). This demonstrates that RIF induces a stress response in *M. tuberculosis* strains which harbor an *rpoB* mutation. Of the 41 proteins identified, 7 (17%) are annotated to be involved in different pathways which are essential for the production of energy. We hypothesize that the up regulation of ATP synthesis enzymes would be essential to support the energy requirements of active efflux which had been previously shown to be a mechanism regulating the intracellular concentration of RIF in these strains (PhD thesis, Gail E. Louw, 2009). Thus our subsequent analysis focused on two of the proteins involved in ATP synthesis, *atpA* and *atpH*. Both of these proteins were weakly present prior to exposure with RIF and their abundance increased significantly in response to RIF. Transcriptomic analysis confirmed that *atpA* and *atpH* were up regulated in the presence of RIF in both strains. However, up regulation of the *atpA* gene was more pronounced in the high level RIF resistant strain (R451) than the intermediate level RIF resistant strain (R439). In contrast, the *atpH* gene was equally up regulated in both strains in the presence of RIF. In order to explain the up regulation of the *atpA* and *atpH*, each gene and the associated up stream regulatory domains were sequenced. No mutations were observed in the ribosome binding sites, while a single synonymous nucleotide polymorphism was observed at codon 91 of the *atpH* gene in both drug resistant and drug susceptible isolates. Therefore, this nucleotide substitution can be classified as a polymorphism. In addition, this polymorphism was found to

be specific for the LCC family and can therefore be used as a valuable tool to identify outbreaks of the LCC family. These genomic markers can aid in the understanding of the evolution and epidemiology of *M. tuberculsosis* strain families (9,16).

AtpA and *atpH* encode for two of the eight subunits that forms part of the final enzyme, F_0F_1 ATP Synthase (complex V), of the oxidative phosphorylation pathway (4). Annotation of the H37Rv genome shows that these genes are adjacently located together with a further six genes (*atpB, atpE, atpF, atpG, atpD, atpC*) (http://genolist.pasteur.fr/TubercuList) suggesting a single operon regulated by a common operator and promoter. This is supported by recent studies which have shown that all 8 of these genes are essential for *in vitro* growth (13) and are down regulated at 24h and 96h of starvation (2),oxygen depletion, NO treatment and during different respiratory states in a murine model of infection (14). Recently it was revealed that two additional genes, Rv1303 and Rv1312 are part of the ATP synthase operon (12). It was also shown that that the binding site of a transcriptional regulator, Rv1846c, is located in a region upstream of the *rv1303–atpBEFHAGDC–rv1312* operon. This suggests that the gene expression of this operon is regulated by Rv1846c. The primary function of F_0F_1 ATP synthase is to convert electrochemical potential energy of protons into chemical energy of ATP phosphoanhydride bonds (6). However, in some organisms the reverse reaction, the hydrolysis of ATP to ADP produces a trans membrane proton pump, is more important (8). Interestingly, one of the subunits of the F_0F_1 ATP synthase, AtpE, was identified as the drug target of a promising new drug for the treatment of TB (11). TMC207 (also known as R207910) is the lead compound of a series of recently discovered diarylquinolines (1). Recent clinical studies confirmed that TMC207 is active *in vitro* against both drug susceptible and drug resistant isolates , thereby confirming the essentiality of this enzyme for bacterial growth (1,5). Subsequently, it was shown that TMC207 decreased the time to smear conversion of MDR-TB cases when used in combination with 2^{nd} line anti-TB drugs (5).

From these findings we proposed a model which might explain how *M. tuberculosis* RIF resistant strains have adapted to survive under high concentrations of RIF. In a drug sensitive strain, the hydrophobic RIF molecule enters the cell through passive diffusion through the membrane. It binds to the beta subunit of the RNA polymerase *(rpoB)*, inhibiting RNA transcription, which results in cell death. Under these conditions a low level of expression of efflux pumps (PhD thesis, Gail E. Louw, 2009) and ATP synthase (This study) was observed. In a RIF resistant strain a mutation in the *rpoB* gene results in a conformational change in the rpoB protein, thereby altering the binding constant between RIF and the

rpoB protein. Thus transcription can proceed and the cell will continue to grow and replicate. Under these conditions we postulated that the accumulation of unbound RIF in the cell will stimulate a stress response which will lead to the up regulation of genes involved in detoxification. These up regulated genes include efflux pumps (PhD thesis, Gail E. Louw, 2009), ATP synthase (this study) and other metabolic enzymes (this study). In this model ATP synthase will serve as an energy source, driving metabolic processes, including membrane ABC transporters. ABC transporters utilize free energy from ATP hydrolysis to pump a variety of compounds, including antibiotics, out of the cell, thereby defining the MIC for RIF (3,10). This model may explain how the inclusion TMC207 in the MDR-TB treatment regimen improves smear conversion. We propose that the inhibition of efflux by the inhibition of ATP synthesis will lead to an accumulation of anti-TB drug within the cell. The high intracellular concentration of anti-TB drug will allow for more efficient binding of the drug to the target and cell death.

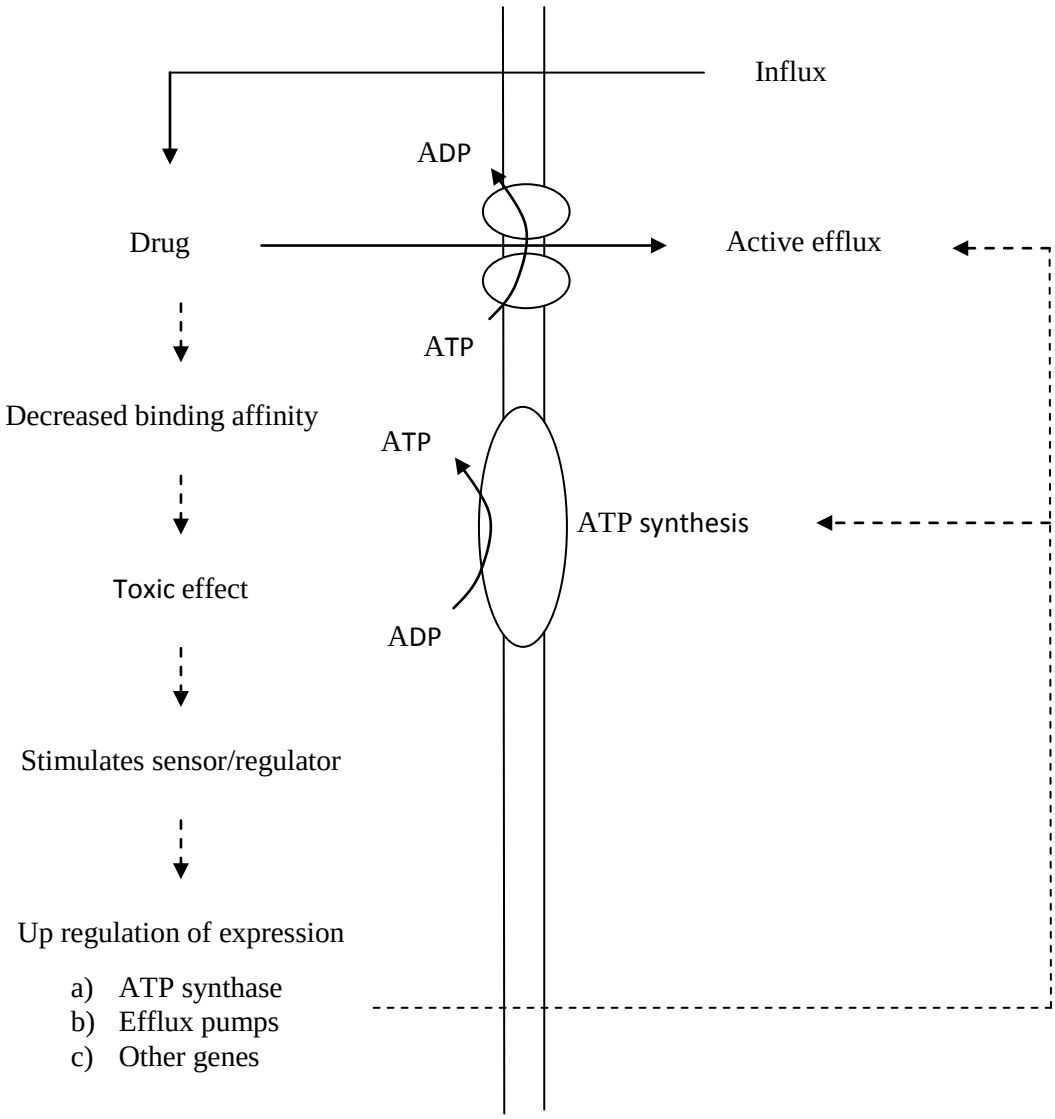

Figure 5.1: Proposed model for the survival of *M. tuberculosis* RIF resistant strains under high concentrations of RIF

Reference List

1. **Andries, K., P. Verhasselt, J. Guillemont, H. W. Gohlmann, J. M. Neefs, H. Winkler, J. Van Gestel, P. Timmerman, M. Zhu, E. Lee, P. Williams, D. de Chaffoy, E. Huitric, S. Hoffner, E. Cambau, C. Truffot-Pernot, N. Lounis, and V. Jarlier**. 2005. A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science **307**:223-227.

2. **Betts, J. C., P. T. Lukey, L. C. Robb, R. A. McAdam, and K. Duncan**. 2002. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol.Microbiol. **43**:717-731.

3. **Choudhuri, B. S., S. Bhakta, R. Barik, J. Basu, M. Kundu, and P. Chakrabarti**. 2002. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes drrA and drrB of Mycobacterium tuberculosis. Biochem.J. **367**:279-285.

4. **ckers-Hebestreit, G. and K. Altendorf**. 1996. The F0F1-type ATP synthases of bacteria: structure and function of the F0 complex. Annu.Rev.Microbiol. **50**:791-824.

5. **Diacon, A. H., A. Pym, M. Grobusch, R. Patientia, R. Rustomjee, L. Page-Shipp, C. Pistorius, R. Krause, M. Bogoshi, G. Churchyard, A. Venter, J. Allen, J. C. Palomino, M. T. De, R. P. van Heeswijk, N. Lounis, P. Meyvisch, J. Verbeeck, W. Parys, B. K. de, K. Andries, and D. F. Mc Neeley**. 2009. The diarylquinoline TMC207 for multidrug-resistant tuberculosis. N.Engl.J.Med. **360**:2397-2405.

6. **Feniouk, B. A., T. Suzuki, and M. Yoshida**. 2006. The role of subunit epsilon in the catalysis and regulation of FOF1-ATP synthase. Biochim.Biophys.Acta **1757**:326-338.

7. **Grove, H., K. Hollung, A. K. Uhlen, H. Martens, and E. M. Faergestad**. 2006. Challenges related to analysis of protein spot volumes from two-dimensional gel electrophoresis as revealed by replicate gels. J.Proteome.Res. **5**:3399-3410.

8. **Kawasaki-Nishi, S., T. Nishi, and M. Forgac**. 2003. Proton translocation driven by ATP hydrolysis in V-ATPases. FEBS Lett. **545**:76-85.

9. **Louw, G. E., R. M. Warren, P. D. van Helden, and T. C. Victor**. 2009. Rv2629 191A/C nucleotide change is not associated with rifampicin resistance in Mycobacterium tuberculosis. Clin.Chem.Lab Med. **47**:500-501.

10. **Pasca, M. R., P. Guglierame, F. Arcesi, M. Bellinzoni, E. De Rossi, and G. Riccardi**. 2004. Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in Mycobacterium tuberculosis. Antimicrob.Agents Chemother. **48**:3175-3178.

11. **Petrella, S., E. Cambau, A. Chauffour, K. Andries, V. Jarlier, and W. Sougakoff**. 2006. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. Antimicrob.Agents Chemother. **50**:2853-2856.

12. **Sala, C., A. Haouz, F. A. Saul, I. Miras, I. Rosenkrands, P. M. Alzari, and S. T. Cole**. 2009. Genome-wide regulon and crystal structure of BlaI (Rv1846c) from Mycobacterium tuberculosis. Mol.Microbiol. **71**:1102-1116.

13. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin**. 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol.Microbiol. **48**:77-84.

14. **Shi, L., C. D. Sohaskey, B. D. Kana, S. Dawes, R. J. North, V. Mizrahi, and M. L. Gennaro**. 2005. Changes in energy metabolism of Mycobacterium tuberculosis in mouse lung and under in vitro conditions affecting aerobic respiration. Proc.Natl.Acad.Sci.U.S.A **102**:15629-15634.

15. **Stessl, M., C. R. Noe, and B. Lachmann**. 2009. Influence of image-analysis software on quantitation of two-dimensional gel electrophoresis data. Electrophoresis **30**:325-328.

16. **Victor, T. C., P. E. de Haas, A. M. Jordaan, G. D. van der Spuy, M. Richardson, D. van Soolingen, P. D. van Helden, and R. Warren**. 2004. Molecular characteristics and global spread of Mycobacterium tuberculosis with a western cape F11 genotype. J.Clin.Microbiol. **42**:769-772.

CHAPTER 6

CONCLUSION

This study highlights the importance of investigating the mechanisms underlying drug resistance in clinical isolates. The originality and novelty of this study further lies in the application of state-of-the-art technologies to test the hypothesis that RIF resistance develops through unique mechanisms in addition to the nsSNP in the *rpoB* gene. In this study a three pronged approach (proteomic, transcriptomic and genomic methods) was used to enhance the possibility of identifying mechanisms which could explain variation in the level of RIF resistance in two closely related clinical isolates which demonstrate identical genetic backgrounds according to standard markers. Using these approaches it was possible to show that RIF induced expression of a number of different membrane proteins in both clinical isolates. Furthermore, it was evident that there was a correlation between the number of proteins up regulated and the level of RIF resistance. Together these results suggest that different strains may use different strategies to survive the toxic nature of RIF.

Many of the up regulated proteins were associated with metabolic processes, in particular, the identification of two proteins, AtpA and AtpH, which may form part of an operon encoding for the FoF1 ATP synthase. The identification of these two proteins in two independent experiments indicates that increased expression of these genes is essential for the survival of the bacterium after RIF exposure. These results were confirmed by QRT-PCR. No mutations involved in the regulation of translation could be identified to explain differential expression of these genes.

By combining these results with other observations from our laboratory it was possible to propose a model to explain at least one mechanism whereby the two clinical isolates were able to regulate the intracellular concentration of RIF. In this model we suggest that RIF induces a toxic response which leads to up regulation of expression of a number of genes including efflux pumps and F_0F_1 ATP synthase. We suggest that the F_0F_1 ATP synthase provides the energy requirement to enable active efflux, thereby regulating the intracellular concentration of RIF. Our results provide the first evidence to suggest that the evolution of RIF resistance is a dynamic process involving a cascade of adaptive events which leads to a bacterial growth state where hydrophobic compounds are actively extruded from the cell. This contrasts significantly from the present dogma which suggests that the evolution of RIF resistance involves only a single mutation which disrupts in the binding of RIF to *rpoB.* If our model is correct this could have significant implications for the treatment of RIF resistant strains with hydrophobic anti-TB drugs as prior exposure to RIF may “condition” the bacillus to extrude these compounds thereby reducing their

therapeutic effect. This may explain why the inclusion of the ATP synthase inhibitor TMC207 in the treatment regimen improved the rate of smear conversion in patients with drug resistant TB. We propose that TMC207 blocked energy production thereby limiting active efflux leading to an increase in the intracellular concentration of anti-TB drugs with a corresponding increase in bactericidal activity.

Future studies need to rigorously test the proposed hypothesis, to establish the mechanisms whereby the cell detects the toxic effect of RIF and to determine the cascade of regulatory events which lead to up regulation of F_0F_1 ATP synthase and numerous efflux pumps.

CHAPTER 7

FUTURE STUDIES

The following future studies are needed to test our proposed model:

1. Determine if the ATP synthase operon are encoded by a single polycistronic DNA. This could be done by PCR amplification of intragenic regions.
2. Determine if there are any internal promoters inside the coding region. This could be done by cloning the intragenic region upstream of a reporter gene and then measuring expression of the reporter in *M. smegmatis*.
3. Determine the location of the ATP synthase operon promoter region by cloning the region upstream of Rv1303 into a "promoter-trap" plasmid.
4. Identify genes that may regulate the expression of the ATP synthase operon using saturating transposon mutagenesis.
5. Use saturating transposon mutagenesis to identify genes which sense the toxic compound (i.e. RIF).
6. Determine whether other hydrophobic anti-TB drugs are able to stimulate up regulation of the ATP synthase operon by QT-PCR.
7. Determine whether exposure of a RIF resistant strain to RIF induces resistance to other hydrophobic anti-TB drugs.
8. Determine the effect of TMC207 on the activity of ABC transporters in drug resistant isolates by measuring the MIC for RIF in the presence of TMC207.